

## METHODS FOR PRODUCING OLFACTORY GPCRS

This application claims the benefit of priority from the following provisional application, filed via U.S. Express mail with the United States Patent and Trademark Office on the indicated date:

- 5 U.S. Provisional Number 60/523,940, filed November 21, 2003. The disclosure of the foregoing application is herein incorporated by reference in its entirety.

### BACKGROUND OF THE INVENTION

#### 10 FIELD OF THE INVENTION

The present invention relates to methods for producing GPCR proteins, particularly olfactory GPCR proteins, in a cell.

#### BACKGROUND OF THE INVENTION

- 15 All animals possess a "nose", an olfactory sense organ that allows for the recognition and discrimination of chemosensory information in the environment. Humans, for example, have a poor sense of smell compared to other animals, and yet they can perceive, i.e., smell, over 10,000 volatile chemicals ("odorants") that are typically small organic molecules of less than 400 Da. These chemicals vary greatly in structure and include a panoply of diverse
- 20 aliphatic acids, alcohols, aldehydes, ketones, and esters; chemicals with aromatic, alicyclic, polycyclic and heterocyclic ring structures; and innumerable substituted chemicals of each of these types, as well as combinations thereof. Remarkably, these molecules are not only detected by the olfactory system, they are discriminated by it.

- Since some odors are desirable and other odors are repulsive, the ability to produce new
- 25 odors, mimic odors, and manipulate perception of odor is extremely desirable. Towards this end, research into odor perception has intensified in recent years. In humans and other animal species, a large number of odorant receptors have been identified on olfactory cilia, a specialized type of dendrite of an olfactory sensory neuron. These odorant receptors exhibit a seven transmembrane domain topology characteristic of the superfamily of G-protein coupled
- 30 receptors, and, accordingly, are termed "olfactory GPCRs". Each olfactory sensory neuron expresses only one type of olfactory GPCR, and it is estimated that the human genome encodes approximately 500 active olfactory GPCRs.

- Accordingly, a vast number of chemicals may be detected and discriminated by a relatively small number of receptors. This is achieved using a combinatorial receptor coding
- 35 scheme in which each olfactory GPCR recognizes more than one odorant, each odorant is

recognized by more than one olfactory GPCR. Thus odorants can be characterized according to the "fingerprint" of activated GPCRs. Once determined, this "fingerprint" provides the identity of the odorant as well as the basis for identification of other molecules that exhibit a similar "fingerprint" and thus smell.

5        Despite the fact that that recombinant olfactory GPCRs can be efficiently expressed at high levels by olfactory sensory neurons *in vivo* when exogenously introduced using an adenoviral vector (e.g., Touhara et al, Proc. Natl. Acad. Sci. 96: 4040-4045, 1999), olfactory GPCRs are quite exceptional in that they cannot be easily expressed in heterologous cultured cell systems in a manner that provides for their function in the cell (e.g., McClintock, Mol.  
10    Brain Res. 48:270-278, 1997). While the exact cause of this problem is not clear, one theory is that when expressed in non-endogenous cells, olfactory GPCRs are not exported to the plasma membrane of the cell and become sequestered in the endoplasmic reticulum. Another theory suggests that functional expression may be due to an olfactory specific factor required for proper membrane localization or inefficient coupling to transduction machinery in non-  
15    olfactory cells (Krieger et al, Eur. J. Biochem 219:829-835, 1994). Regardless of the mechanism that results in the inefficient and/or essentially non-functional expression of recombinant olfactory GPCRs in non-endogenous cultured cells, the solution to the problem has been previously unknown.

20        Until the present invention, olfactory GPCRs were exceptionally difficult to express in mammalian cells *in vitro*, and, even though such methods are extremely desirable, there were no robust, reliable and efficient method for producing and assaying those GPCRs. Progress in understanding odorant perception and discrimination has been severely hampered because olfactory GPCRs cannot be produced (Firestein Nature 413:211-218, 2001).

25        It follows from the foregoing that there is a great need for robust, reliable and efficient methods for producing olfactory GPCRs in a mammalian cell. This invention meets this need, and others, with unpredictably high level of success.

#### LITERATURE

30        Literature of interest includes the following references: Zozulya et al, (Genome Biology 2:0018.1-0018.12, 2001; Mombairts (Annu. Rev. Neurosci 22:487-509, 1999); Raming et al, (Nature 361: 353-356, 1993); Belluscio et al, (Neuron 20: 69-81, 1988); Ronnet et al, (Annu. Rev. Physiol. 64:189-222, 2002); Lu et al, (Traffic 4: 416-533, 2003); Buck (Cell 100:611-618, 2000); Malnic et al, (Cell 96:713-723, 1999); Firestein (Nature 413:211-218, 2001); Zhao et al, (Science 279: 237-242, 1998); Touhara et al, (Proc. Natl. Acad. Sci. 96: 4040-4045, 1999); Sklar et al, (J. Biol. Chem 261:15538-15543, 1986); Dryer et al, (TiPS 20:413-417, 1999); Ivic  
35    et al, (J Neurobiol. 50:56-68, 2002); and Fuchs et al, (Hum. Genet. 108:1-13, 2001); published

US patent applications 20030143679 and 20030105285; and US patents 6,610,511, 6,492,143 and 6,410,249.

#### SUMMARY OF THE INVENTION

5           The subject invention provides a method for producing an olfactory GPCR in a cell. In general, the methods involve introducing an expression cassette containing a promoter operably linked to a nucleic acid encoding an olfactory GPCR into a macroglial cell, e.g., a Schwann or oligodendritic cell, and maintaining the cell under conditions suitable for production of the olfactory GPCR. Also provided is a macroglial cell containing a recombinant nucleic acid  
10       encoding an olfactory GPCR, methods of screening for modulators of olfactory GPCR activity, and a kit for producing an olfactory GPCR in a macroglial cell. The invention finds most use in research on flavors and fragrances, and, consequently, has a variety of research and industrial applications.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15           **FIG. 1** is four panels of photographs showing expression of recombinant human olfactory GPCR on the surface of primary rat Schwann cells. Panel OR1 is olfactory GPCR having Genbank accession number P47893. Panel OR2 is olfactory GPCR having Genbank accession number NP\_036505. Panel OR3 is olfactory GPCR having Genbank accession  
20       number XP\_166868. Vector is empty expression vector negative control. Olfactory GPCR is expressed from a CMV promoter-based expression vector as an N-terminal fusion protein comprising a rhodopsin signal peptide and a hemagglutinin (HA) epitope tag.

#### DEFINITIONS

25           Before the present invention is further described, it is to be understood that this invention is not limited to particular embodiments described, as such may of course vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of  
30       ordinary skill in the art to which this invention belongs.

          Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges  
35       may independently be included in the smaller ranges, and are also encompassed within the

invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Throughout this application, various publications, patents and published patent  
5 applications are cited. The disclosures of these publications, patents and published patent applications referenced in this application are hereby incorporated by reference in their entirety into the present disclosure. Citation herein by Applicant of a publication, patent, or published patent application is not an admission by Applicant of said publication, patent, or published patent application as prior art.

10 It must be noted that as used herein and in the appended claims, the singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an agent" includes a plurality of such agents, and reference to "the GPCR" includes reference to one or more GPCRs and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any  
15 optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

"G-protein coupled receptors", or "GPCRs" are polypeptides that share a common structural motif, having seven regions of between 22 to 24 hydrophobic amino acids that form  
20 seven alpha helices, each of which spans a membrane [each span is identified by number, *i.e.*, transmembrane-1 (TM1), transmembrane-2 (TM2), *etc.*]. The transmembrane helices are joined by regions of amino acids between transmembrane-2 and transmembrane-3, transmembrane-4 and transmembrane-5, and transmembrane-6 and transmembrane-7 on the exterior, or "extracellular" side, of the cell membrane [these are referred to as "extracellular" regions 1, 2  
25 and 3 (EC1, EC2 and EC3), respectively]. The transmembrane helices are also joined by regions of amino acids between transmembrane-1 and transmembrane-2, transmembrane-3 and transmembrane-4, and transmembrane-5 and transmembrane-6 on the interior, or "intracellular" side, of the cell membrane [these are referred to as "intracellular" regions 1, 2 and 3 (IC1, IC2 and IC3), respectively]. The "carboxy" ("C") terminus of the receptor lies in the intracellular  
30 space within the cell, and the "amino" ("N") terminus of the receptor lies in the extracellular space outside of the cell. GPCR structure and classification is generally well known in the art, and further discussion of GPCRs may be found in Probst, DNA Cell Biol. 1992 11:1-20; Marchese et al Genomics 23: 609-618, 1994; and the following books: Jürgen Wess (Ed) Structure-Function Analysis of G Protein-Coupled Receptors published by Wiley-Liss (1st  
35 edition; October 15, 1999); Kevin R. Lynch (Ed) Identification and Expression of G Protein-

Coupled Receptors published by John Wiley & Sons (March 1998) and Tatsuya Haga (Ed), G Protein-Coupled Receptors, published by CRC Press (September 24, 1999); and Steve Watson (Ed) G-Protein Linked Receptor Factsbook, published by Academic Press (1st edition; 1994).

5 A "native GPCR" is a GPCR that is produced by an animal, e.g., a mammal such as a human or mouse. Detailed description of native GPCRs may be found in the On-line Mendelian Inheritance in Man database found at the world wide website of the National Center of Biotechnology Information (NCBI). Additional description of native GPCRs may be found at the world wide website of primalinc.com and a list of exemplary GPCRs for use in the subject methods is set forth in Table 1.

10 The term "ligand" means a molecule that specifically binds to a GPCR. A ligand may be, for example a polypeptide, a lipid, a small molecule or an antibody, etc. A "native ligand" is a ligand that is an endogenous, natural ligand for a native GPCR. A ligand may be a GPCR "antagonist", "agonist", "partial agonist" or "inverse agonist", or the like.

A "modulator" is a ligand that increases or decreases a GPCR intracellular response  
15 when it is in contact with, e.g., binds, to a GPCR that is expressed in a cell.

The term "second messenger" shall mean an intracellular response produced as a result of receptor activation. A second messenger can include, for example, inositol 1,4,5-triphosphate (IP3), diacylglycerol (DAG), cyclic AMP (cAMP), cyclic GMP (cGMP), and Ca<sup>2+</sup>. Second messenger response can be measured for a determination of receptor activation.  
20 In addition, second messenger response can be measured for the identification of candidate agents as, for example, agonists, partial agonists, inverse agonists, and antagonists.

An "agonist" is a ligand which activates a GPCR intracellular response when it binds to a GPCR.

A "partial agonist" is a ligand what activates, to a lesser extent than an agonist, a GPCR  
25 intracellular response when it binds to a GPCR.

An "antagonist" is a ligand which competitively binds to a GPCR at the same site as an agonist but which does not activate the intracellular response produced by the active form of a GPCR. Antagonists usually inhibit intracellular responses by an agonist or partial agonist. Antagonists usually do not diminish the baseline intracellular response in the absence of an  
30 agonist or partial agonist.

An "inverse agonist" is a ligand which binds to a GPCR and inhibits the baseline (basal) intracellular response of the GPCR observed in the absence of an agonist or partial agonist. In most embodiments, a baseline intracellular response is inhibited in the presence of an inverse agonist by at least about 30%, by at least about 50%, or by at least 75%, as compared to a  
35 baseline response in the absence of an inverse agonist.

The term "odorant" encompasses any compound, naturally occurring or chemically synthesized, of known or unknown structure, that activates an olfactory GPCR. As discussed in the Background section above, odorants are usually volatile, small organic molecules of less than 400 Da. Flavors, perfumes, scents, odors, fragrance are types of odorants. An "odor" is the sensation associated with a particular odorant.

The term "phenomenon associated with olfactory GPCR activity" as used herein refers to a structural, molecular, or functional characteristic associated with olfactory GPCR activity, particularly such a characteristic that is readily assessable in a human or animal model. Such characteristics include, but are not limited to, downstream molecular events caused by activation of a GPCR, and sensory phenotypes such as smell, taste, or other behavioral or physiological events caused by activation of a GPCR.

A "deletion" is defined as a change in either amino acid or nucleotide sequence in which one or more amino acid or nucleotide residues, respectively, are absent as compared to an amino acid sequence or nucleotide sequence of a parental GPCR polypeptide or nucleic acid. In the context of a GPCR or a fragment thereof, a deletion can involve deletion of about 2, about 5, about 10, up to about 20, up to about 30 or up to about 50 or more amino acids. A GPCR or a fragment thereof may contain more than one deletion.

An "insertion" or "addition" is that change in an amino acid or nucleotide sequence which has resulted in the addition of one or more amino acid or nucleotide residues, respectively, as compared to an amino acid sequence or nucleotide sequence of a parental GPCR. "Insertion" generally refers to addition to one or more amino acid residues within an amino acid sequence of a polypeptide, while "addition" can be an insertion or refer to amino acid residues added at an N- or C-terminus, or both termini. In the context of a GPCR or fragment thereof, an insertion or addition is usually of about 1, about 3, about 5, about 10, up to about 20, up to about 30 or up to about 50 or more amino acids. A GPCR or fragment thereof may contain more than one insertion.

A "substitution" results from the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively as compared to an amino acid sequence or nucleotide sequence of a parental GPCR or a fragment thereof. It is understood that a GPCR or a fragment thereof may have conservative amino acid substitutions which have substantially no effect on GPCR activity. By conservative substitutions is intended combinations such as gly, ala; val, ile, leu; asp, glu; asn, gln; ser, thr; lys, arg; and phe, tyr.

The term "biologically active" GPCR refers to a GPCR having structural and biochemical functions of a naturally occurring GPCR.

As used herein, the terms “determining,” “measuring,” “assessing,” and “assaying” are used interchangeably and include both quantitative and qualitative determinations. Reference to an “amount” of a GPCR in these contexts is not intended to require quantitative assessment, and may be either qualitative or quantitative, unless specifically indicated otherwise.

5       The terms “polypeptide” and “protein”, used interchangeably herein, refer to a polymeric form of amino acids of any length, which can include coded and non-coded amino acids, chemically or biochemically modified or derivatized amino acids, and polypeptides having modified peptide backbones. The term includes fusion proteins, including, but not limited to, fusion proteins with a heterologous amino acid sequence, fusions with heterologous and homologous leader sequences, with or without N-terminal methionine residues;  
10       immunologically tagged proteins; fusion proteins with detectable fusion partners, e.g., fusion proteins including as a fusion partner a fluorescent protein,  $\beta$ -galactosidase, luciferase, etc.; and the like.

      The terms “nucleic acid molecule” and “polynucleotide” are used interchangeably and  
15       refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. Non-limiting examples of polynucleotides include a gene, a gene fragment, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides,  
20       plasmids, vectors, isolated DNA of any sequence, control regions, isolated RNA of any sequence, nucleic acid probes, and primers. The nucleic acid molecule may be linear or circular.

      As used herein the term “isolated,” when used in the context of an isolated compound, refers to a compound of interest that is in an environment different from that in which the compound naturally occurs. “Isolated” is meant to include compounds that are within samples  
25       that are substantially enriched for the compound of interest and/or in which the compound of interest is partially or substantially purified.

      As used herein, the term “substantially pure” refers to a compound that is removed from its natural environment and is at least 60% free, preferably 75% free, and most preferably 90% free from other components with which it is naturally associated.

30       A “coding sequence” or a sequence that “encodes” a selected polypeptide, is a nucleic acid molecule which can be transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide, for example, in a host cell when placed under the control of appropriate regulatory sequences (or “control elements”). The boundaries of the coding sequence are typically determined by a start codon at the 5' (amino) terminus and a translation  
35       stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to,

cDNA from viral, procaryotic or eucaryotic mRNA, genomic DNA sequences from viral or prokaryotic DNA, and synthetic DNA sequences. A transcription termination sequence may be located 3' to the coding sequence. Other "control elements" may also be associated with a coding sequence. A DNA sequence encoding a polypeptide can be optimized for expression in a selected cell by using the codons preferred by the selected cell to represent the DNA copy of the desired polypeptide coding sequence.

"Encoded by" refers to a nucleic acid sequence which codes for a polypeptide sequence, wherein the polypeptide sequence or a portion thereof contains an amino acid sequence of at least 3 to 5 amino acids, more preferably at least 8 to 10 amino acids, and even more preferably at least 15 to 20 amino acids from a polypeptide encoded by the nucleic acid sequence. Also encompassed are polypeptide sequences that are immunologically identifiable with a polypeptide encoded by the sequence.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. A promoter that is operably linked to a coding sequence will effect the expression of a coding sequence. The promoter or other control elements need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. For example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

By "nucleic acid construct" it is meant a nucleic acid sequence that has been constructed to comprise one or more functional units not found together in nature. Examples include circular, linear, double-stranded, extrachromosomal DNA molecules (plasmids), cosmids (plasmids containing COS sequences from lambda phage), viral genomes comprising non-native nucleic acid sequences, and the like.

A "vector" is capable of transferring gene sequences to a host cell. Typically, "vector construct," "expression vector," and "gene transfer vector," mean any nucleic acid construct capable of directing the expression of a gene of interest and which can transfer gene sequences to host cells, which can be accomplished by genomic integration of all or a portion of the vector, or transient or inheritable maintenance of the vector as an extrachromosomal element. Thus, the term includes cloning, and expression vehicles, as well as integrating vectors.

An "expression cassette" comprises any nucleic acid construct capable of directing the expression of a gene/coding sequence of interest, which is operably linked to a promoter of the expression cassette. Such cassettes can be constructed into a "vector," "vector construct," "expression vector," or "gene transfer vector," in order to transfer the expression cassette into a host cell. Thus, the term includes cloning and expression vehicles, as well as viral vectors.



A first polynucleotide is "derived from" or "corresponds to" a second polynucleotide if it has the same or substantially the same nucleotide sequence as a region of the second polynucleotide, its cDNA, complements thereof, or if it displays sequence identity as described above.

5 A first polypeptide is "derived from" or "corresponds to" a second polypeptide if it is (i) encoded by a first polynucleotide derived from a second polynucleotide, or (ii) displays sequence identity to the second polypeptides as described above.

The terms "administering", and the like, refers to adding a GPCR modulatory agent to obtain a desired pharmacologic and/or physiologic effect. In many embodiments, the subject  
10 GPCR modulatory agents are volatile, and, as such, they are administered orally or intranasally, either directly or indirectly by addition to foodstuffs or to the atmosphere. The effect may completely or partially prevent perception of an odor, may increase perception of an odorant, or may generate a new odor.

The term "non-naturally occurring" or "recombinant" means artificial or otherwise not  
15 found in nature. Recombinant cells usually contain nucleic acid that is not usually found in that cell, recombinant nucleic acid usually contain a fusion of two or more nucleic acids that is not found in nature, and a recombinant polypeptide is usually produced by a recombinant nucleic acid.

"Subject", "individual," "host" and "patient" are used interchangeably herein, to refer to  
20 any animal, e.g., mammal, human or non-human, having olfactory GPCRs. Generally, the subject is a mammalian subject. Exemplary subjects include, but are not necessarily limited to, humans, non-human primates, mice, rats, cattle, sheep, goats, pigs, dogs, cats, and horses, with humans being of particular interest.

## 25 DETAILED DESCRIPTION OF THE INVENTION

The subject invention provides a method for producing an olfactory GPCR in a cell. In general, the methods involve introducing an expression cassette containing a promoter operably linked to a nucleic acid encoding an olfactory GPCR into a macroglial cell, e.g., a Schwann or oligodendritic cell, and maintaining the cell under conditions suitable for production of the  
30 olfactory GPCR. Also provided is a macroglial cell containing a recombinant nucleic acid encoding an olfactory GPCR, methods of screening for modulators of olfactory GPCR activity, and a kit for producing an olfactory GPCR in a macroglial cell. The invention finds use in, for example, analysis and identification of flavors and fragrances, and, consequently, has a variety of research and industrial applications.

In further describing the invention in greater detail than provided in the Summary and as informed by the Background and Definitions provided above, methods of producing an olfactory GPCR are described first, followed by a description of compositions and kits that find use in performing the subject methods. Finally, methods of screening for modulators of olfactory GPCR activity and methods of screening for odorant mimetics are discussed.

#### METHODS FOR PRODUCING AN OLFACTORY GPCR

In one aspect, the invention provides methods of producing an olfactory GPCR in a cell. In describing these methods, the compositions for use in the methods will be described first.

##### *Olfactory G-protein coupled receptors*

The term "olfactory G-protein coupled receptor" (or abbreviations thereof, e.g., "olfactory GPCR") refers to any member of a phylogenetically distinct, art-recognized subfamily of the GPCR superfamily that is involved in chemosensation. Olfactory GPCRs are both generally and specifically disclosed in a wide variety of publications and public databases, including Zozulya et al, (Genome Biol. 2:0018, 2001); Glusman et al, (Genome Res. 11: 685-702, 2001) and Crasto et al, (Nucleic Acids Res. 30:354-60, 2002), which are specifically incorporated herein in their entirety. In particular, the olfactory GPCRs set forth in the database of olfactory GPCR sequences found at the world wide website of the Senselab.med.yale.edu are of interest. A non-limiting list of exemplary olfactory GPCRs suitable for use in the subject methods is provided in Table 1, inserted before the claims. Table 1 is a list of accession numbers of protein sequence entries from the Swiss-Prot database, as found at the world wide website of the European Bioinformatics Institute. These database entries listed in Table 1, in particular the amino acids sequences set forth in those entries, are specifically incorporated herein by reference in their entirety.

It is expressly contemplated that the olfactory GPCR may be of human origin or of non-human animal origin. In certain embodiments, the non-human animal may be a mouse, a rat, a dog, or any other non-human animal with an acute and discriminating sense of smell. In certain embodiments, the olfactory GPCR may be of insect origin (e.g., mosquito, ant, aphid, beetle, fly, wasp, bee, spider, or any insect which transmits a disease to human or non-human animals or which causes damage to crops or ornamental plants). In particular embodiments, the olfactory GPCR is human.

It is recognized that both native and altered native olfactory GPCRs may be used in the subject methods. Accordingly, the term "olfactory G-protein coupled receptor" is also intended to encompass an altered native olfactory GPCR (e.g. a native olfactory GPCR that is altered by

addition such as an addition of a reporter, substitution, deletions and insertions, etc.) such that it binds the same ligand as a corresponding native GPCR.

The term "olfactory G-protein coupled receptor" therefore includes variants of the GPCR polypeptides recited in Table 1. In other words, variants of any olfactory GPCR may be used in the subject methods. In certain embodiments, therefore, an olfactory GPCR may have an altered sequence as compared to a native sequence (e.g., a sequence deposited in NCBI's Genbank database or the like). For example, an olfactory GPCR may be a native polypeptide having any number of amino acid substitutions, amino acid deletions, or amino acid additions at any position in the polypeptide (e.g., the C- or N-terminus, or at internal positions).

In particular embodiments, the olfactory GPCR is a fusion protein, and may contain, for example, an affinity tag domain or a reporter domain. Suitable affinity tags include any amino acid sequence that may be specifically bound to another moiety, usually another polypeptide, most usually an antibody. Suitable affinity tags include epitope tags, for example, the V5 tag, the FLAG tag, the HA tag (from hemagglutinin influenza virus), the myc tag, and the like, as is known in the art. Suitable affinity tags also include domains for which, binding substrates are known, e.g., HIS, GST and MBP tags, as is known in the art, and domains from other proteins for which specific binding partners, e.g., antibodies, particularly monoclonal antibodies, are available. Suitable affinity tags also include any protein-protein interaction domain, such as a IgG Fc region, which may be specifically bound and detected using a suitable binding partner, e.g. the IgG Fc receptor. It is expressly contemplated that such a fusion protein may contain a heterologous N-terminal domain (e.g., an epitope tag) fused in-frame with a GPCR that has had its N-terminal methionine residue either deleted or substituted with an alternative amino acid. In certain embodiments, the olfactory GPCR fusion protein may comprise at its N-terminus a rhodopsin signal peptide alone or in combination with a hemagglutinin epitope tag. In particular embodiments, the olfactory GPCR fusion protein may comprise an N-terminus having the amino acid sequence MNGTEGPNFYVPFSNKTGVVYPYDVPDYAKL, where MNGTEGPNFYVPFSNKTGVV is rhodopsin signal peptide and YPYDVPDYAKL is hemagglutinin epitope tag. It is well within the purview of persons of skill in the art to construct an expression cassette allowing for the expression of the olfactory GPCR as a fusion protein (see, e.g., Krautwurst et al, Cell 95:917-926, 1998). It is appreciated that a polypeptide of interest may first be made from a native polypeptide and then operably linked to a suitable reporter/tag as described above. In other embodiments, an olfactory GPCR may be a fragment of a GPCR, wherein said GPCR fragment is biologically active.

Suitable reporter domains include any domain that can report the presence of a polypeptide. While it is recognized that an affinity tag may be used to report the presence of a

polypeptide using, e.g., a labeled antibody that specifically binds to the tag, light emitting reporter domains are more usually used. Suitable light emitting reporter domains include luciferase (from, e.g., firefly, *Vargula*, *Renilla reniformis* or *Renilla muelleri*), and light emitting variants thereof. Other suitable reporter domains include fluorescent proteins (from  
5 e.g., jellyfish, corals and other coelenterates as such those from *Aequoria*, *Renilla*, *Ptilosarcus*, *Stylatula* species), or light emitting variants thereof. Light emitting variants of these reporter proteins are very well known in the art and may be brighter, dimmer, or have different excitation and/or emission spectra, as compared to a native reporter protein. For example, some variants are altered such that they no longer appear green, and may appear blue, cyan, yellow,  
10 enhanced yellow red (termed BFP, CFP, YFP eYFP and RFP, respectively) or have other emission spectra, as is known in the art. Other suitable reporter domains include domains that can report the presence of a polypeptide through a biochemical or color change, such as  $\beta$ -galactosidase,  $\beta$ -glucuronidase, chloramphenicol acetyl transferase, and secreted embryonic alkaline phosphatase. In some preferred embodiments, the reporter domain is *Renilla* luciferase  
15 (e.g., pRLCMV; Promega, catalog number E2661).

Also, as is known in the art, an affinity tags or a reporter domain may be present at any position in an olfactory GPCR. However, in most embodiments, they are present at the C- or N-terminal end of an olfactory GPCR.

In many embodiments, an olfactory GPCR is a member of a library of olfactory GPCRs.  
20 Typically, a library contains a plurality of members, where a plurality may be 2 or more, 5 or more, about 10 or more, about 20 or more, about 50 or more, about 100 or more, about 200 or more, about 300 or more, about 500 or more, about 1000 or more, or even up to about 10,000 or more. The library may therefore contain about 5, about 10, about 20, about 30 or more, about 50 or more, about 100 or more, about 200 or more, usually up to 500 or more, usually up to  
25 about 1000 or more olfactory GPCR polypeptides. The members of the library may be of known identity, or unknown identity, or a mixture thereof. The members of the library may be entirely derived from one species or may be derived from a plurality of species.

#### *Nucleic acids encoding olfactory G-protein coupled receptors*

Since the genetic code and recombinant techniques for manipulating nucleic acid are  
30 known, and the amino acid sequences of olfactory GPCR polypeptides are described above, the design and production of nucleic acids encoding an olfactory GPCR polypeptide is well within the skill of an artisan. In certain embodiments, standard recombinant DNA technology (Ausubel, et al, *Short Protocols in Molecular Biology*, 3rd ed., Wiley & Sons, 1995; Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (1989) Cold Spring Harbor,  
35 N.Y.) methods are used. For example, olfactory GPCR coding sequences may be isolated from

a library of olfactory GPCR coding sequence using any one or a combination of a variety of recombinant methods that do not need to be described herein in any great detail. Subsequent substitution, deletion, and/or addition of nucleotides in the nucleic acid sequence encoding a protein may also be done using standard recombinant DNA techniques.

5 For example, site directed mutagenesis and subcloning may be used to introduce/delete/substitute nucleic acid residues in a polynucleotide encoding a polypeptide of interest. In other embodiments, PCR may be used. Nucleic acids encoding a polypeptide of interest may also be made by chemical synthesis entirely from oligonucleotides (e.g., Cello et al., *Science* (2002) 297:1016-8).

10 In certain embodiments, the codons of the nucleic acids encoding polypeptides of interest are optimized for expression in cells of a particular species, particularly a mammalian, e.g., human or mouse species.

The invention further provides vectors (also referred to as "constructs") comprising a subject nucleic acid. In many embodiments of the invention, the subject nucleic acid sequences  
15 will be expressed in a host after the sequences have been operably linked to an expression control sequence, including, e.g. a promoter to form an expression cassette. A subject expression cassette is typically placed in an expression vector that can replicate in a host cell either as an episome or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit  
20 detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Pat. No. 4,704,362, which is incorporated herein by reference). Vectors, including single and dual expression cassette vectors are well known in the art (Ausubel, et al, *Short Protocols in Molecular Biology*, 3rd ed., Wiley & Sons, 1995; Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (1989) Cold Spring Harbor, N.Y.). Suitable vectors  
25 include viral vectors, plasmids, cosmids, artificial chromosomes (human artificial chromosomes, bacterial artificial chromosomes, yeast artificial chromosomes, etc.), mini-chromosomes, and the like. Retroviral, adenoviral and adeno-associated viral vectors may be used.

A variety of expression vectors are available to those in the art for purposes of  
30 producing a polypeptide of interest in a cell. One suitable vector is pCMV, which used in certain embodiments. This vector was deposited with the American Type Culture Collection (ATCC) on October 13, 1998 (10801 University Blvd., Manassas, VA 20110-2209 USA) under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure. The DNA was tested by the ATCC and

determined to be viable. The ATCC has assigned the following deposit number to pCMV:  
ATCC #203351.

5 The subject expression cassettes usually comprise a single open reading frame encoding an olfactory GPCR, however, in certain embodiments, since the host cell for expression of the olfactory GPCR may be a eukaryotic cell, e.g., a mammalian cell, such as a human cell, the open reading frame may be interrupted by introns. Subject expression cassettes are typically part of a transcriptional unit which may contain, in addition to the subject nucleic acid 3' and 5' untranslated regions (UTRs) which may direct RNA stability, translational efficiency, etc. The expression cassette may also be part of a nucleic acid which contains, in addition to the subject  
10 nucleic acid, a transcriptional terminator.

The subject expression cassettes may comprise nucleic acid sequence allowing for expression of the olfactory GPCR as a fusion protein. In certain embodiments, the olfactory GPCR fusion protein may comprise at its N-terminus a rhodopsin signal peptide and/or a hemagglutinin epitope tag. In particular embodiments, the olfactory GPCR fusion protein may  
15 comprise an N-terminus having the amino acid sequence  
MNGTEGPNFYVPFSNKTGVVYPYDVPDYAKL,  
where MNGTEGPNFYVPFSNKTGVV is rhodopsin signal peptide and  
YPYDVPDYAKL is hemagglutinin epitope tag. It is well within the purview of persons of skill in the art to construct an expression cassette allowing for the expression of the olfactory  
20 GPCR as a fusion protein (see, e.g., Krautwurst et al, Cell 95:917-926, 1998).

Eukaryotic promoters (i.e., promoters that function in a eukaryotic cell) can be any promoter that is functional in a macroglial cell, including viral promoters and promoters derived from eukaryotic genes. Exemplary eukaryotic promoters include, but are not limited to, the following: the promoter of the mouse metallothionein I gene sequence (Hamer et al., J. Mol.  
25 Appl. Gen. 1:273-288, 1982); the TK promoter of Herpes virus (McKnight, Cell 31:355-365, 1982); the SV40 early promoter (Benoit et al., Nature (London) 290:304-310, 1981); the yeast gall gene sequence promoter (Johnston et al., Proc. Natl. Acad. Sci. (USA) 79:6971-6975, 1982; Silver et al., Proc. Natl. Acad. Sci. (USA) 81:5951-5955, 1984), the CMV promoter, the EF-1 promoter, Ecdysone-responsive promoter(s), tetracycline-responsive promoter, and the  
30 like. Viral promoters may be of particular interest as they are generally particularly strong promoters. In certain embodiments, a promoter is used that is a viral promoter. Promoters for use in the present invention are selected such that they are functional in the macroglial cells (and/or animal) into which they are being introduced. In certain embodiments, the promoter is a CMV promoter.

In certain embodiments, a subject vector may also provide for expression of a selectable marker. Suitable vectors and selectable markers are well known in the art and discussed in Ausubel, et al, (Short Protocols in Molecular Biology, 3rd ed., Wiley & Sons, 1995) and Sambrook, et al, (Molecular Cloning: A Laboratory Manual, Third Edition, (2001) Cold Spring Harbor, N.Y.). A variety of different genes have been employed as selectable markers, and the particular gene employed in the subject vectors as a selectable marker is chosen primarily as a matter of convenience. Known selectable marker genes include: the thymidine kinase gene, the dihydrofolate reductase gene, the xanthine-guanine phosphoribosyl transferase gene, CAD, the adenosine deaminase gene, the asparagine synthetase gene, the antibiotic resistance genes, e.g. tetr, ampr, Cmr or cat, kanr or neor (aminoglycoside phosphotransferase genes), the hygromycin B phosphotransferase gene, and the like.

As mentioned above, olfactory GPCRs may be fusion proteins that contain an affinity domain and/or a reporter domain. Methods for making fusions between a reporter or tag and a GPCR, for example, at the C- or N-terminus of the GPCR, are well within the skill of one of skill in the art (e.g. McLean et al, Mol. Pharma. Mol Pharmacol. 1999 56:1182-91; Ramsay et al., Br. J. Pharmacology, 2001, 315-323) and will not be described any further. It is expressly contemplated that such a fusion protein may contain a heterologous N-terminal domain (e.g., an epitope tag) fused in-frame with a GPCR that has had its N-terminal methionine residue either deleted or substituted with an alternative amino acid. It is appreciated that a polypeptide of interest may first be made from a native polypeptide and then operably linked to a suitable reporter/tag as described above.

The subject nucleic acids may also contain restriction sites, multiple cloning sites, primer binding sites, ligatable ends, recombination sites etc., usually in order to facilitate the construction of a nucleic acid encoding an olfactory GPCR.

Since an olfactory GPCR may be member of a library of polypeptides of interest, the nucleic acids encoding such a polypeptide of interest may also be a similar sized library of nucleic acids encoding olfactory GPCRs.

#### *Host cells*

The methods described herein generally involve producing an olfactory GPCR in a cultured macroglial cell (i.e., a primary or immortal macroglial cell cultured *in vitro*). By "macroglial cell" is meant any cell of a variety of neuron-associated cell types, including: Schwann cells, oligodendrocytes and astrocytes, and derivatives thereof. In many embodiments, suitable host cells may be "myelin-producing" cells that produce myelin, the material that forms sheath of nerve axons. Myelin-producing macroglial cells include Schwann cells, oligodendrocytes, as well as certain types of astrocytes that produce myelin (e.g., olfactory

sheathing cells). Myelin producing cells can usually be identified by their synthesis of a galactocerebroside, gal C, which is a component of myelin.

Also encompassed by the term "macroglial cells" are modified versions of macroglial cells, including cancerous macroglial cells, e.g., Schwannoma, neurofibromas, astrocytoma cells, and oligodendrocytoma cells; immortal macroglial cells, e.g., cells immortalized via introduction of a suitable oncogenes, e.g., HPV E6-E7, T antigen, and the like; hybrid cells produced by cell fusion in which a macroglial cell is fused with a different (non-macroglial) or a like (macroglial) type of cell; and recombinant macroglial cells, e.g., cells that have contain an exogenous nucleic acid, or a "knockout" in an endogenous gene, e.g., a gene required for or that inhibits the synthesis of myelin. Macroglial cells are usually from mammalian species, such as rodents (e.g., mouse) or humans. Exemplary and non-limiting cell lines include RN2 and EJ (Coulter-Mackie, Virus Research 1:477-487, 1984), RN22 (Kreider, Brain Research 397:238-244, 1986), and HOG and MO3.13 (Buntinx, Journal of Neurocytology 32:25-38, 2003). A macroglial cell recombinant for other than an olfactory GPCR is expressly contemplated to be encompassed by the term "macroglial cell."

Accordingly, since methods of culturing macroglial cells are well known in the art, (see, e.g., Mosahebi Glia, 34:8-17, 2001; Shen, Microsurgery 19:356-63, 1999; Acta Neuropathol (Berl), 78:317-24, 1989; Barnett, Developmental Biology, 155: 337-350, 1993; and Hung et al, International Journal of Oncology 20: 475-482, 2002) a variety of suitable host cells are available for production of olfactory GPCRs, including immortalized HEI193 cells and the like.

In particular, Schwann cells may be cultured using the following methods: Hung, (Int. J. Oncol. 20:475-82, 2002); Hung, (Int. J. Oncol. 1999 14:409-15); Wood, (Brain Res. 115:361-75, 1976); Wood, (Ann. N.Y. Acad. Sci. 605:1-14, 1990); and Brockes, (J. Exp. Biol. Dec;95:215-30, 1981).

Additional cell lines will become apparent to those of ordinary skill in the art, and are available from the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209.

#### *Methods*

In general accordance with the subject methods, an olfactory GPCR expression cassette is introduced into a macroglial cell *in vitro*, the cell is subjected to conditions suitable for expression of the olfactory GPCR, and the GPCR is expressed in the cell and exported to the cell surface.

Accordingly, in most embodiments, an expression cassette may be introduced into a host cell using a variety of methods, including viral infection, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate precipitation, direct microinjection, and



the like. The choice of method is generally dependent on the type of cell being transformed and the circumstances under which the transformation is taking place (e.g., *in vitro*, etc.). A general discussion of these methods can be found in Ausubel, et al, Short Protocols in Molecular Biology, 3rd ed., Wiley & Sons, 1995.

5           After introduction of an expression cassette for an olfactory GPCR into a cell, the cell is typically incubated to provide for polypeptide expression. To accomplish this, the cell may be incubated in suitable media for 12-24 hr, 24-48 hr, or 48-96 hr or more. Transient expression of the polypeptide may be carried out in this manner. It is expressly contemplated, however, that expression of the polypeptide may alternatively be stable. In stable transfection, the expression  
10           cassette contains a selectable marker gene and establishment of a stable cell line expressing the polypeptide involves selection for the selectable marker gene. If two expression cassettes are introduced into a cell, the two expression cassettes usually contain two different selectable marker genes (e.g., neomycin resistance gene and hygromycin resistance gene). Methods of transient and stable transfection are well known to those of skill in the art.

15           Olfactory GPCRs are produced in the macroglial cell, and usually exported to the surface of the cell such that the GPCR is present in the plasma membrane.

#### COMPOSITIONS

20           In another aspect, the invention provides a macroglial cell producing a biologically active olfactory GPCR. Such cells usually contain a recombinant nucleic acid encoding an olfactory GPCR, and may produce an olfactory GPCR that is not usually produced in that cell (i.e. a macroglial cell in the absence of the recombinant nucleic acid).

25           As mentioned above, the present invention provides a macroglial cell containing an olfactory GPCR that is present (i.e., detectably present) at the surface of the macroglial cell, usually spanning the plasma membrane of the cell in a manner that is characteristic of GPCRs. Accordingly, the subject cells contain "active" olfactory GPCRs in that they are capable of binding a ligand, and transmitting a signal via a suitable G-protein, if present. The subject cells thus find use in activity assays, e.g., screening assays, which will be described in great detail below.

30           The subject cells usually produce olfactory GPCR at a significantly level greater than that of control cells such as a non-macroglial cells, e.g. an NIH-3T3 cell, COS cell, or the like, into which the same expression cassette has been produced. In most embodiments, the subject cells produce, on a molar basis, at least 5x ("5 times"), at least 10x, at least 50x, at least 100x, usually up to at least 1000x more olfactory GPCR than control cells. In particular embodiments,  
35           the subject cells produce, on a molar basis, at least 5x ("5 times"), at least 10x, at least 50x, at

least 100x, usually up to at least 1000x more olfactory GPCR at the cell surface than control cells (e.g., as determined by immunocytochemistry or flow cytometry). When the subject cells are grown in liquid culture, they usually produce olfactory GPCR in significant amounts, e.g., greater than 10 $\mu$ g/l, greater than 100 $\mu$ g/l, greater than 1mg/l, greater than 10mg/l or greater than  
5 about 50mg/l or more. In particular, when the subject cells are grown in liquid culture, they usually produce cell surface olfactory GPCR in significant amounts, e.g., greater than 10 $\mu$ g/l, greater than 100 $\mu$ g/l, greater than 1mg/l, greater than 10mg/l or greater than about 50mg/l or more.

Since there are a number of different olfactory GPCRs, the invention also provides a  
10 plurality of macroglial cells (i.e., a library of macroglial cells) containing a corresponding plurality of recombinant nucleic acids encoding different olfactory GPCRs. In these embodiments, each macroglial cell of the plurality usually contains a recombinant nucleic acid for a single olfactory GPCR, and each cell contains a different nucleic acid. Accordingly, the invention provides a library of macroglial cells, the cells containing recombinant nucleic acids  
15 encoding 2 or more, 5 or more, about 10 or more, about 20 or more, about 50 or more, about 100 or more, about 200 or more, about 300 or more, about 500 or more, about 1000 or more different olfactory GPCRs. The olfactory GPCRs may be of known identity, or unknown identity, or a mixture thereof. The olfactory GPCRs may be derived from a single species or alternatively derived from 2, up to about 5, up to about 10, up to about 50, up to about 100, or  
20 up to about 1000 species of animal. In certain embodiments, the olfactory GPCRs are human.

#### KITS

Also provided by the subject invention are kits for practicing the subject methods, as described above. The subject kits at least include one or more of: a macroglial cell, a nucleic  
25 acid encoding an olfactory GPCR, and a macroglial cell containing an olfactory GPCR. The nucleic acids of the kit may also have restrictions sites, multiple cloning sites, primer sites, etc to facilitate their ligation into other plasmids. Other optional components of the kit include: culture media, components for testing GPCR activity, and G-protein-encoding nucleic acids, etc, for performing the subject assays. The various components of the kit may be present in  
30 separate containers or certain compatible components may be precombined into a single container, as desired.

In addition to above-mentioned components, the subject kits typically further include instructions for using the components of the kit to practice the subject methods, e.g., methods of producing an olfactory GPCR, etc. The instructions for practicing the subject methods are  
35 generally recorded on a suitable recording medium. For example, the instructions may be

printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or subpackaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

#### METHODS FOR IDENTIFYING MODULATORS OF OLFACTORY GPCR ACTIVITY

The invention provides methods of screening for olfactory GPCR modulators (i.e., compounds that increase or decrease the activity of an olfactory GPCR of interest). In certain embodiments, the olfactory GPCR modulator is selected from the group consisting of agonist, partial agonist, inverse agonist, and antagonist. In general, the methods involve producing an olfactory GPCR in a macroglial cell according to the methods described above to provide a macroglial cell producing a biologically active olfactory GPCR (termed herein a "subject macroglial cell"), contacting the cell with a candidate agent, and assessing the effect of the candidate agent on an activity of the olfactory GPCR.

In other embodiments, a modulator of an olfactory GPCR (e.g. a natural or synthetic ligand for that GPCR, for example) may be contacted with a macroglial producing that GPCR, and the effect of the modulator on the activity of the olfactory GPCR may be assessed. Also envisioned are assays that are done using two olfactory GPCR modulators, e.g., an activator of an olfactory GPCR (for example, a ligand for that GPCR), and an agent that blocks the modulatory activity of the activator.

As is known in the art, the subject assays may be performed using a variety of methods, such as, for example, membrane binding assays using  $^{35}\text{S}$  GTP $\gamma$ S, adenylyl cyclase assays (e.g., using the FLASH PLATE<sup>TM</sup> Adenylyl Cyclase kit from New England Nuclear; Cat. No. SMP004A), cell-based cAMP assays, reporter-based assays, AP1 reporter assays, SRF-LUC reporter assays, intracellular IP3 accumulation assays, fluorometric imaging plate reader (FLIPR) assays for the measurement of intracellular calcium concentration, and the like.

In embodiments where the modulator increases olfactory GPCR activity, the activity of an olfactory GPCR is increased in the presence of the modulator by at least about 10%, by at least about 20%, by at least about 30%, by at least about 50%, by at least about 80%, by at least

about 100%, by at least about 500%, or by at least about 10-fold or more, as compared to suitable controls in the absence of the agent. Suitable controls may be in the presence or absence of the native ligand for the GPCR.

5 In embodiments where the modulator decreases olfactory GPCR activity, the activity of the olfactory GPCR is decreases in the presence of the modulator by at least about 10%, by at least about 20%, by at least about 30%, by at least about 50%, by at least about 70%, by at least about 80%, by at least about 90%, or by at least about 95% or more, as compared to suitable controls in the absence of the agent. Suitable controls may be in the presence or absence of the native ligand for the GPCR.

10 In certain embodiments, these methods also involve measuring GPCR activity in the presence or absence of a test compound, e.g., a candidate agent. These assays may involve contacting an isolated subject macroglial cell (e.g., a cultured cell), a membrane isolated from a subject macroglial cell, an extract of a subject macroglial cell, with an amount of a GPCR modulator that is effective to modulate the activity of the GPCR.

15 Accordingly, the invention provides for inhibitors of olfactory GPCR activity to reduce the activity of an olfactory GPCR in the presence or absence of a ligand, e.g., a natural ligand, for that GPCR, and inducers of GPCR activity, where the GPCR is induced by a compound that is or is not the natural ligand of the GPCR.

In certain embodiments, GPCR activity may be measured by assessing a reporter signal.  
20 In these embodiments, the assays may be performed in a format suitable for high throughput assays, e.g., 96- or 384- well format, and suitable robots, (e.g., pipetting robots), and instrumentation (96- or 384- well format luminometers or fluorescence readers for determining reporter activity) may be used. By way of illustration and not limitation, determining reporter activity may employ a Wallac 1450 Microbeta counter (Perkin-Elmer) or a CCD camera-based  
25 illuminator.

In related embodiments, the assay may be a binding assay, wherein the binding of a candidate agent to an olfactory GPCR is assessed. In these embodiments, the candidate agent is usually first labeled, contacted with a subject macroglial cell, and binding of the agent to the macroglial cell assessed.

### 30 *Candidate agents*

A variety of different test compounds may be screened by the above methods. Test compounds encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds (i.e., compounds having a molecular weight of more than 50 and less than about 2,500 daltons (e.g, 100-1000 Da, usually less than about 500  
35 Da)). Test compounds comprise functional groups necessary for structural interaction with

proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The test compounds often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Exemplary  
5 /and non-limiting test compounds include aliphatic acids, alcohols, ketones, and esters; chemicals with aromatic, alicyclic, polycyclic and heterocyclic ring structures; and innumerable substituted chemicals of each of these types, as well as combinations thereof. Test compounds are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Further test  
10 compounds include variants of a GCPR's native ligand.

Test compounds may be obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural  
15 compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. A library may preferentially comprise natural or synthetically produced compounds associated with smell. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to  
20 directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, *etc.* to produce structural analogs.

Of interest are test compounds that are polypeptides, e.g., proteinaceous, agents. A specific type of polypeptide test compound of interest is an antibody for the GPCR, or a GPCR-binding fragment thereof. The antibody may be monoclonal or polyclonal, and may be  
25 produced according to methods known in the art. Further test compounds include variants of the GCPR's native ligand, for a GPCR having a known native ligand, e.g. a native ligand that is altered by substitution, deletion or addition of at least one amino acid, or chemically modified. In certain embodiments test compounds include endogenous polypeptides not known to be ligands of the GPCR.

30 The foregoing characterization of test compounds is intended to be illustrative and not limiting.

## **METHODS FOR IDENTIFYING A CANDIDATE AGENT AS A LIGAND OF AN OLFACTORY GPCR.**

5 A ligand of an olfactory GPCR may be identified by contacting a candidate agent with the olfactory GPCR and determining whether the candidate agent binds to the olfactory GPCR, wherein said binding is indicative of the candidate agent being a ligand of the olfactory GPCR. In certain embodiments, the candidate agent may be labeled. In particular embodiments, the candidate agent may be radiolabeled.

10 Suitable radionuclides that may be incorporated into subject candidate agents include but are not limited to  $^2\text{H}$  (deuterium),  $^3\text{H}$  (tritium),  $^{11}\text{C}$ ,  $^{13}\text{C}$ ,  $^{14}\text{C}$ ,  $^{13}\text{N}$ ,  $^{15}\text{N}$ ,  $^{15}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ,  $^{18}\text{F}$ ,  $^{35}\text{S}$ ,  $^{36}\text{Cl}$ ,  $^{82}\text{Br}$ ,  $^{75}\text{Br}$ ,  $^{76}\text{Br}$ ,  $^{77}\text{Br}$ ,  $^{123}\text{I}$ ,  $^{124}\text{I}$ ,  $^{125}\text{I}$  and  $^{131}\text{I}$ . Incorporation of  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{82}\text{Br}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$  or may generally be most useful.

Synthetic methods for incorporating radio-isotopes into organic compounds are applicable to subject candidate agents and are well known in the art. These synthetic methods, 15 for example, incorporating activity levels of tritium into target molecules, are as follows:

A. Catalytic Reduction with Tritium Gas - This procedure normally yields high specific activity products and requires halogenated or unsaturated precursors.

20 B. Reduction with Sodium Borohydride [ $^3\text{H}$ ] - This procedure is rather inexpensive and requires precursors containing reducible functional groups such as aldehydes, ketones, lactones, esters, and the like.

C. Reduction with Lithium Aluminum Hydride [ $^3\text{H}$ ] - This procedure offers products at almost theoretical specific activities. It also requires precursors containing reducible functional groups such as aldehydes, ketones, lactones, esters, and the like.

25 D. Tritium Gas Exposure Labeling - This procedure involves exposing precursors containing exchangeable protons to tritium gas in the presence of a suitable catalyst.

E. N-Methylation using Methyl Iodide [ $^3\text{H}$ ] - This procedure is usually employed to prepare O-methyl or N-methyl ( $^3\text{H}$ ) products by treating appropriate precursors with high specific activity methyl iodide ( $^3\text{H}$ ). This method in general allows for higher specific activity, such as for example, about 70-90 Ci/mmol.

30 Synthetic methods for incorporating activity levels of  $^{125}\text{I}$  into target molecules include:

A. Sandmeyer and like reactions - This procedure transforms an aryl or heteroaryl amine into a diazonium salt, such as a tetrafluoroborate salt, and subsequently to  $^{125}\text{I}$  labeled compound using  $\text{Na}^{125}\text{I}$ . A represented procedure was reported by Zhu, D.-G. and co-workers in *J. Org. Chem.* 2002, 67, 943-948.

B. Ortho <sup>125</sup>Iodination of phenols – This procedure allows for the incorporation of <sup>125</sup>I at the ortho position of a phenol as reported by Collier, T. L. and co-workers in *J. Labeled Compd Radiopharm.* 1999, 42, S264-S266.

C. Aryl and heteroaryl bromide exchange with <sup>125</sup>I – This method is generally a two step process. The first step is the conversion of the aryl or heteroaryl bromide to the corresponding tri-alkyltin intermediate using for example, a Pd catalyzed reaction [i.e. Pd(Ph<sub>3</sub>P)<sub>4</sub>] or through an aryl or heteroaryl lithium, in the presence of a tri-alkyltinhalide or hexaalkylditin [e.g., (CH<sub>3</sub>)<sub>3</sub>SnSn(CH<sub>3</sub>)<sub>3</sub>]. A represented procedure was reported by Bas, M.-D. and co-workers in *J. Labeled Compd Radiopharm.* 2001, 44, S280-S282.

A ligand of an olfactory GPCR may alternatively be identified by contacting a candidate agent with the olfactory GPCR in the presence of a labeled known ligand of the olfactory GPCR, wherein a decrease of binding of the labeled known ligand in the presence of the candidate agent is indicative of the candidate agent being a ligand of the olfactory GPCR.

#### METHODS FOR IDENTIFYING ODORANT MIMETICS

The invention also provides methods for identifying odorant mimetics, where a mimetic is a synthetic or natural chemical compound that has similar, substantially the same or identical functional characteristics as a particular odorant, but has a different chemical structure to the odorant. In other words, the invention provides methods of identifying an odorant mimetic that “smells” the same as an odorant of interest, but does not have the same chemical structure as the odorant of interest. In general, these methods involve producing a library of olfactory GPCRs using the methods set forth above, identifying a set of olfactory GPCRs that are activated by an odorant of interest, and contacting the library of olfactory GPCRs with candidate agents to identify an agent that activates the same set of olfactory GPCRs. In most embodiments, an agent that activates the same set of olfactory GPCRs as an odorant of interest is a mimetic of the odorant of interest, i.e., should have a similar odor to the odorant of interest.

Accordingly, these methods usually involve producing a library (e.g., 100 or more, 200 or more, 300 or more, 400 or more, 500 or more, 600 or more, usually up to about 1000 or more) different olfactory GPCRs using the methods described above, and assessing the GPCRs to determine whether they are activated by an odorant of interest, e.g., a compound of known or unknown chemical structure that has a desirable smell or taste. In many embodiments, the odorant of interest will activate a set of olfactory GPCRs, where a set usually contains 2-50, 2-20 or 3-10 members. The set of olfactory GPCRs activated by a single odorant provides a “GPCR fingerprint”, where a single odorant is defined by the set of olfactory GPCRs that it activates. A mimetic for an odorant of interest may be identified by screening a library of

candidate agents to identify an agent that has an identical or near identical GPCR fingerprint to that of the odorant of interest.

For example, an odorant mimetic can be identified so that it has a "fingerprint" of activated GPCRs similar to that of the odorant of interest, e.g., the mimetic activates about 5 60%, about 75%, about 80%, about 90%, about 95% of the GPCRs or GPCR activity as that activated by the odorant.

Accordingly, mimetics of an odorant of interest may be identified.

#### BIOSENSING METHODS

10 The invention also provides a biosensor, where the biosensor is typically a plurality of macroglial cells producing a plurality of different olfactory GPCRs. In many embodiments, the cells are arrayed in an addressable format, wherein each address of the array contains macroglial cells producing a single recombinant olfactory GPCR. Typically, said plurality may be 2 or more, 5 or more, about 10 or more, about 20 or more, about 50 or more, about 100 or 15 more, about 200 or more, about 300 or more, about 500 or more, about 1000 or more, or even up to about 10,000 or more. The biosensor may therefore contain about 5, about 10, about 20, about 30 or more, about 50 or more, about 100 or more, about 200 or more, usually up to 500 or more, usually up to about 1000 or more recombinant olfactory GPCRs. The olfactory GPCRs may be of known identity, or unknown identity, or a mixture thereof. The olfactory GPCRs may 20 be derived from a single species or alternatively derived from 2, up to about 5, up to about 10, up to about 50, up to about 100, or up to about 1000 species of animal. In certain embodiments, the olfactory GPCRs are human.

The methods described herein involve binding of said macroglial cells producing a single recombinant olfactory GPCR to an "affinity substrate". In certain embodiments, said 25 affinity substrate is addressable. In particular embodiments, said addressable affinity substrate is spatially addressable. An affinity substrate contains a solid, semi-solid, or insoluble support and is made from any material appropriate for binding of said recombinant macroglial cells and does not interfere with the detection method used. As will be appreciated by those in the art, the number of possible affinity substrates is very large. Possible substrates include, but are not 30 limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, ceramics, and a variety of other polymers. In a preferred embodiment, the substrates 35 allow optical detection and do not themselves appreciably fluoresce or emit light. In addition, as



is known the art, the substrate may be coated with any number of materials, including polymers, such as dextrans, acrylamides, gelatins, agarose, biocompatible substances such as proteins including bovine and other mammalian serum albumin.

5 A "spatially addressable" affinity substrate has multiple, discrete, regions (e.g., multiple polypeptide of interest-binding regions) such that each region is at a particular predetermined location (an "address"). Multi-well microtiter plates are addressable (each well having an address), an array of capillary columns is addressable, an array of samples deposited onto a solid support (e.g., a nylon or nitrocellulose membrane) is addressable. Affinity substrates for use in the methods described herein typically have at least 4 or more, at least about 12, at least  
10 about 24, at least about 48, at least about 96 or at least about 384 or addressable regions. In particular embodiments, an affinity substrate is in an addressable format suitable for high throughput assays, e.g., a 24-, 48- 96- or 384- well format.

Such multi-well formats are suitable for use by robots, (e.g., pipetting robots), and other instrumentation (96- or 384- well format luminometers or fluorescence readers for determining  
15 reporter activity). By way of illustration and not limitation, reporter activity may be measured using a CCD camera-based illuminator.

In use, such biosensors are usually contacted with a sample, and activation of each of the recombinant olfactory GPCRs is assessed. The presence of an odorant of interest is detected by activation of a pre-determined subset of olfactory GPCRs, where the pre-  
20 determined subset of olfactory GPCRs corresponds to a previously determined "GPCR fingerprint" of that odorant. Accordingly, if a pre-determined subset of GPCRs for an odorant of interest is activated by the sample, then an odorant of interest is present in the sample.

In alternative use, such biosensors are usually contacted with an odorant, and activation of each of the recombinant GPCRs is assessed. Identification of a "fingerprint" for that odorant  
25 is assigned based on the subset of olfactory GPCRs activated. In a variation of said alternative use, said contacting may be carried out in the presence or more or more agonists to the olfactory GPCRs of the biosensor, with the subset of GPCRs activated by the odorant in the presence of said one more agonists representing another means of assigning a "fingerprint" to an odorant. It is envisioned that in the presence of an agonist, inverse agonist or antagonist activity of one or  
30 more olfactory GPCRs may be incorporated into a "fingerprint" of an odorant.

In certain embodiments, GPCR activation may be detected using a light-emitting reporter of GPCR activation. For example, any light-emitting reporter (e.g., a fluorescent reporter, etc.) assay may be used such as the luciferase/GFP based assays described below, or variations thereof, may be used for these assays.

In certain embodiments, the activation of one or more of the olfactory GPCRs to an odorant may be scored as being at a particular level, such as by exemplification and not limitation 0-10%, 11-25%, 26-50%, 51-75%, or 76-100% of a pre-determined maximum response. It is envisioned that a "fingerprint" of an odorant may be determined at least in part  
5 by the level of activation of one or more of the olfactory GPCRs.

Accordingly, the invention provides a light-emitting biosensor that contains an addressable array of macroglial cells containing olfactory GPCRs, where an odorant of interest may be detected by emission of a particular pattern of light from the biosensor.

In certain embodiments, the sample to be tested is an environmental test sample, e.g., a  
10 sample of a gas (such as a sample of a breathable atmosphere or a gas of unknown origin or composition), liquid (such as a sample of water or a liquid of unknown origin or composition), or any solid.

The biosensor methods described above find particular use in, for example: crime-scenes, where knowledge of a smell, for example, may lead to capture of a suspect for a crime;  
15 war zones (e.g., battlefields), where certain chemicals, e.g., biological/chemical warfare agents, may be detected; foodstuffs, where, e.g., certain contaminants or desirable or undesirable smells can be detected; and in the rational assignment of a particular olfactory GPCR or a particular subset of olfactory GPCR to either a desirable or undesirably olfactory sensation; and in laboratories where it is desirable to monitor noxious chemicals; and, in general, in any situation  
20 in which it is desirable to monitor or detect an odorant of interest.

Odorants of interest generally include any compound that can be detected by the olfactory GPCRs of the human olfactory system, e.g., any compound that can be detected by smelling. The odorant may be a purified compound or may be unpurified (e.g., of complex composition). Such odorants include, but are not limited to, aliphatic acids, alcohols, ketones,  
25 and esters; chemicals with aromatic, alicyclic, polycyclic and heterocyclic ring structures; and innumerable substituted chemicals of each of these types, as well as combinations thereof.

#### UTILITY

The subject methods of producing an olfactory GPCR find use in a variety of research  
30 and commercial applications, particularly those relating to food and fragrance.

In many applications, an item, e.g., a food or fragrance, may be improved, i.e., made more or less desirable, as needed, by addition of an olfactory GPCR modulatory agent identified using the methods described above. In general, such a modulator is usually mixed with the item, e.g., a foodstuff or fragrance such as a perfume, to improve the taste or smell of  
35 the item. In many embodiments, the modulator may be an inhibitor of olfactory GPCR activity,

and therefore "mask" an unpleasant taste or smell. In other embodiments, the modulator may be an activator of certain olfactory GPCRs; and may be used to improve or add a new flavor or fragrance to the matter to which it is added. In other embodiments, the modulator may be an activator of certain olfactory GPCRs, and may be used to improve the efficacy of pesticides. In certain embodiments, it is advantageous to make the odor of some items such as poisons and medicines less desirable so that they are not accidentally consumed. In this case, an agent that provides an unpleasant odor may be discovered by the methods described above and added to those items.

In other applications, the cost of providing a desirable odorant (e.g., an odorant obtained from certain rare flowers and used as a starting material for many of today's perfumes) may be reduced by identifying mimetics of that odorant using the methods described above. In such embodiments, these mimetics may be manufactured at a price that is substantially less than that of the desirable odorant, and may be used to supplement, or replace the desirable odorant in an item, e.g., a perfume, etc.

In other applications, detection of a particular odorant produced by an individual may have diagnostic or prognostic value as relates to a disease or disorder, wherein the elevation or reduction of the particular odorant has been associated with said disease or disorder.

Of course, a variety of individuals may be administered the olfactory GPCR modulators obtained using the methods described above. Generally such individuals are mammals or mammalian, where these terms are used broadly to describe organisms which are within the class mammalia, including the orders carnivore (e.g., dogs and cats), rodentia (e.g., mice, guinea pigs, and rats), and primates (e.g., humans, chimpanzees, and monkeys), as well as mammals of commercial interest such as cows, sheep, pigs and horses. It is envisioned that non-mammalian animals may also be administered the olfactory GPCR modulators obtained using the methods described above. Exemplary and non-limiting non-mammalian animals include birds (e.g., chicken), reptiles, fish, arthropods, and insects (e.g., mosquito, ant, aphid, beetle, fly, wasp, bee, spider, or any insect which transmits a disease to human or non-human animals or which causes damage to crops or ornamental plants). In many embodiments, the individuals will be humans.

#### EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed.

While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the subject invention.

### EXAMPLE 1

#### EXPRESSION OF OLFACTORY GPCRS IN SCHWANN CELLS

##### Primary rat Schwann cell isolation:

The preparation of Schwann cells was done as described previously (e.g., Hung, Int. J. Oncol. 20:475-82, 2002; Hung, Int. J. Oncol. 1999 14:409-15; Wood, Brain Res. 115:361-75, 1976; Wood, Ann. N.Y. Acad. Sci. 605:1-14, 1990; and Brookes, J. Exp. Biol. Dec;95:215-30, 1981, etc). Briefly, sciatic nerves from P1 rat neonates were harvested and cells were maintained in Dulbecco's modified Eagle media supplemented with 10% heat-inactivated fetal bovine serum. Schwann cells were expanded with 2uM forskolin and bovine pituitary extract (Sigma). The cells were grown until the third passage and frozen for storage.

##### Transient transfection of Olfactory GPCRs:

Schwann cells at passage 5 were plated on poly-D-lysine coated 8-well chamber slides (Falcon) at  $8 \times 10^4$  cells per well. The Schwann cells were transfected with 0.5ug of olfactory GPCR expression plasmid with Fugene6 reagent (Roche) and Optimem serum-free medium (Invitrogen). The transfected cells were kept at  $37^{\circ}$  in 5% CO<sub>2</sub> humidified incubator for four hours. Cells were washed with PBS and replaced with fresh growth media. After 24 hours, the cells were assayed for expression.

##### Expression analysis of Olfactory GPCRs determined by HA staining:

The transfected cells were washed with PBSCM (PBS+ 0.5mM Ca<sup>2+</sup> + 1mM MgCl<sub>2</sub>) and fixed with 4% Formalin. Cells were quenched with 50mM NH<sub>4</sub>Cl/ PBSCM and washed twice. Primary antibody anti-mouse HA (Roche) was diluted 1:1000 in blocking buffer (2% BSA in PBSCM w/o triton) and left on cells for 1 hour. After three washes with PBSCM, secondary antibody (Alexa 488-conjugated donkey anti-mouse IgG) 1:2000 and DAPI 1:2000 were left on cells for thirty minutes in the dark. Cells were washed 3 times with PBSCM and coverslipped with flourosave (Calbiochem) Cells analyzed by appropriate UV filters.

Cells producing olfactory GPCR on their surface were observed (see Fig. 1).

## EXAMPLE 2

### GPCR ACTIVATION ASSAYS

*Receptor Expression:* Transient transfection of macroglial cells may be carried out as described in Example 1 for primary rat Schwann cells. Stable transfection of a macroglial cell line may be carried out as described here.

Approximately  $12 \times 10^6$  macroglial cells are plated on a 15cm tissue culture plate and grown in DME High Glucose Medium containing ten percent fetal bovine serum and one percent sodium pyruvate, L-glutamine, and antibiotics. Twenty-four hours following plating of the macroglial cells (or to ~80% confluency), the cells are transfected using 12 $\mu$ g of DNA. The 12 $\mu$ g of DNA is combined with 60 $\mu$ l of lipofectamine and 2mL of DME High Glucose Medium without serum. The medium is aspirated from the plates and the cells are washed once with medium without serum. The DNA, lipofectamine, and medium mixture are added to the plate along with 10ml of medium without serum. Following incubation at 37 degrees Celsius for four to five hours, the medium is aspirated and 25ml of medium containing serum is added. Twenty-four hours following transfection, the medium is aspirated again, and fresh medium with serum is added. Forty-eight hours following transfection, the medium is aspirated and medium with serum is added containing geneticin (G418 drug) at a final concentration of 500 $\mu$ g/ml. The transfected cells now undergo selection for positively transfected cells containing the G418 resistant gene. The medium is replaced every four to five days as selection occurs. During selection, cells are grown to create stable pools, or split for stable clonal selection.

*Membrane Binding Assays: [ $^{35}$ S]GTP $\gamma$ S Assay:* When a G protein-coupled receptor is in its active state, either as a result of ligand binding or constitutive activation, the receptor couples to a G protein and stimulates the release of GDP and subsequent binding of GTP to the G protein. The alpha subunit of the G protein-receptor complex acts as a GTPase and slowly hydrolyzes the GTP to GDP, at which point the receptor normally is deactivated. Activated receptors continue to exchange GDP for GTP. The non-hydrolyzable GTP analog, [ $^{35}$ S]GTP $\gamma$ S, can be utilized to demonstrate enhanced binding of [ $^{35}$ S]GTP $\gamma$ S to membranes expressing activated receptors. The advantage of using [ $^{35}$ S]GTP $\gamma$ S binding to measure activation is that: (a) it is generically applicable to all G protein-coupled receptors; (b) it is proximal at the membrane surface making it less likely to pick-up molecules which affect the intracellular cascade.

The assay utilizes the ability of G protein coupled receptors to stimulate [ $^{35}$ S]GTP $\gamma$ S binding to membranes expressing the relevant receptors. The assay can, therefore, be used in

the direct identification method to screen candidate compounds to endogenous GPCRs and non-endogenous, constitutively activated GPCRs. The assay is generic and has application to drug discovery at all G protein-coupled receptors.

5 The [ $^{35}\text{S}$ ]GTP $\gamma$ S assay is incubated in 20 mM HEPES and between 1 and about 20mM MgCl $_2$  (this amount can be adjusted for optimization of results, although 20mM is preferred) pH 7.4, binding buffer with between about 0.3 and about 1.2 nM [ $^{35}\text{S}$ ]GTP $\gamma$ S (this amount can be adjusted for optimization of results, although 1.2 is preferred ) and 12.5 to 75  $\mu\text{g}$  membrane protein (this amount can be adjusted for optimization) and 10  $\mu\text{M}$  GDP (this amount can be changed for optimization) for 1 hour. Wheatgerm agglutinin beads (25  $\mu\text{l}$ ; Amersham) are then  
10 added and the mixture incubated for another 30 minutes at room temperature. The tubes are then centrifuged at 1500 x g for 5 minutes at room temperature and then counted in a scintillation counter.

*Adenylyl Cyclase A Flash Plate*<sup>TM</sup> Adenylyl Cyclase kit (New England Nuclear; Cat. No. SMP004A) designed for cell-based assays can be modified for use with crude plasma  
15 membranes. The Flash Plate wells can contain a scintillant coating which also contains a specific antibody recognizing cAMP. The cAMP generated in the wells can be quantitated by a direct competition for binding of radioactive cAMP tracer to the cAMP antibody. The following serves as a brief protocol for the measurement of changes in cAMP levels in whole cells that express the receptors.

20 Transfected cells were harvested approximately twenty four hours after transient transfection. Media is carefully aspirated off and discarded. 10ml of PBS is gently added to each dish of cells followed by careful aspiration. 1ml of Sigma cell dissociation buffer and 3ml of PBS are added to each plate. Cells were pipetted off the plate and the cell suspension was collected into a 50ml conical centrifuge tube. Cells were then centrifuged at room temperature  
25 at 1,100 rpm for 5 min. The cell pellet was carefully re-suspended into an appropriate volume of PBS (about 3ml/plate). The cells were then counted using a hemocytometer and additional PBS was added to give the appropriate number of cells (with a final volume of about 50  $\mu\text{l}$ /well).

cAMP standards and Detection Buffer (comprising 1  $\mu\text{Ci}$  of tracer [ $^{125}\text{I}$  cAMP (50  $\mu\text{l}$ ) to  
30 11 ml Detection Buffer) was prepared and maintained in accordance with the manufacturer's instructions. Assay Buffer was prepared fresh for screening and contained 50 $\mu\text{l}$  of Stimulation Buffer, 3 $\mu\text{l}$  of test compound (12 $\mu\text{M}$  final assay concentration) and 50 $\mu\text{l}$  cells, Assay Buffer was stored on ice until utilized. The assay was initiated by addition of 50 $\mu\text{l}$  of cAMP standards to appropriate wells followed by addition of 50 $\mu\text{l}$  of PBSA to wells H-11 and H12. 50 $\mu\text{l}$  of

Stimulation Buffer was added to all wells. DMSO (or selected candidate compounds) was added to appropriate wells using a pin tool capable of dispensing 3 $\mu$ l of compound solution, with a final assay concentration of 12 $\mu$ M test compound and 100 $\mu$ l total assay volume. The cells were then added to the wells and incubated for 60 min at room temperature. 100 $\mu$ l of  
5 Detection Mix containing tracer cAMP was then added to the wells. Plates were then incubated additional 2 hours followed by counting in a Wallac MicroBeta scintillation counter. Values of cAMP/well were then extrapolated from a standard cAMP curve which was contained within each assay plate.

Cell-Based cAMP for Gi Coupled Target GPCRs: TSHR is a Gs coupled GPCR that  
10 causes the accumulation of cAMP upon activation. TSHR will be constitutively activated by mutating amino acid residue 623 (i.e., changing an alanine residue to an isoleucine residue). A Gi coupled receptor is expected to inhibit adenylyl cyclase, and, therefore, decrease the level of cAMP production, which can make assessment of cAMP levels challenging. An effective  
15 technique for measuring the decrease in production of cAMP as an indication of constitutive activation of a Gi coupled receptor can be accomplished by co-transfecting, most preferably, non-endogenous, constitutively activated TSHR (TSHR-A623I) (or an endogenous, constitutively active Gs coupled receptor) as a "signal enhancer" with a Gi linked target GPCR to establish a baseline level of cAMP. Upon creating a non-endogenous version of the Gi  
20 coupled receptor, this non-endogenous version of the target GPCR is then co-transfected with the signal enhancer, and it is this material that can be used for screening. We will utilize such approach to effectively generate a signal when a cAMP assay is used; this approach is preferably used in the direct identification of candidate compounds against Gi coupled receptors. It is noted that for a Gi coupled GPCR, when this approach is used, an inverse  
25 agonist of the target GPCR will increase the cAMP signal and an agonist will decrease the cAMP signal.

On day one,  $2 \times 10^4$  macroglial cells/well will be plated out. On day two, two reaction tubes will be prepared (the proportions to follow for each tube are per plate): tube A will be prepared by mixing 2 $\mu$ g DNA of each receptor transfected into the mammalian cells, for a total of 4 $\mu$ g DNA (e.g., pCMV vector; pCMV vector with mutated TSHR (TSHR-A623I); TSHR-  
30 A623I and GPCR, etc.) in 1.2ml serum free DMEM (Irvine Scientific, Irvine, CA); tube B will be prepared by mixing 120 $\mu$ l lipofectamine (Gibco BRL) in 1.2ml serum free DMEM. Tubes A and B will then be admixed by inversions (several times), followed by incubation at room temperature for 30-45min. The admixture is referred to as the "transfection mixture". Plated macroglial cells will be washed with 1XPBS, followed by addition of 10ml serum free DMEM.

2.4ml of the transfection mixture will then be added to the cells, followed by incubation for 4hrs at 37°C/5% CO<sub>2</sub>. The transfection mixture will then be removed by aspiration, followed by the addition of 25ml of DMEM/10% Fetal Bovine Serum. Cells will then be incubated at 37°C/5% CO<sub>2</sub>. After 24hr incubation, cells will then be harvested and utilized for analysis.

5 A Flash Plate™ Adenylyl Cyclase kit (New England Nuclear; Cat. No. SMP004A) is designed for cell-based assays, however, can be modified for use with crude plasma membranes depending on the need of the skilled artisan. The Flash Plate wells will contain a scintillant coating which also contains a specific antibody recognizing cAMP. The cAMP generated in the wells can be quantitated by a direct competition for binding of radioactive cAMP tracer to the  
10 cAMP antibody. The following serves as a brief protocol for the measurement of changes in cAMP levels in whole cells that express the receptors.

Transfected cells will be harvested approximately twenty four hours after transient transfection. Media will be carefully aspirated off and discarded. 10ml of PBS will be gently added to each dish of cells followed by careful aspiration. 1ml of Sigma cell dissociation buffer  
15 and 3ml of PBS will be added to each plate. Cells will be pipetted off the plate and the cell suspension will be collected into a 50ml conical centrifuge tube. Cells will then be centrifuged at room temperature at 1,100 rpm for 5 min. The cell pellet will be carefully re-suspended into an appropriate volume of PBS (about 3ml/plate). The cells will then be counted using a hemocytometer and additional PBS is added to give the appropriate number of cells (with a  
20 final volume of about 50µl/well).

cAMP standards and Detection Buffer (comprising 1 µCi of tracer [125I cAMP (50 µl] to 11 ml Detection Buffer) will be prepared and maintained in accordance with the manufacturer's instructions. Assay Buffer should be prepared fresh for screening and contained 50µl of Stimulation Buffer, 3µl of test compound (12µM final assay concentration) and 50µl  
25 cells. Assay Buffer can be stored on ice until utilized. The assay can be initiated by addition of 50µl of cAMP standards to appropriate wells followed by addition of 50µl of PBSA to wells H-11 and H12. Fifty µl of Stimulation Buffer will be added to all wells. Selected compounds (e.g., TSH) will be added to appropriate wells using a pin tool capable of dispensing 3µl of compound solution, with a final assay concentration of 12µM test compound and 100µl total  
30 assay volume. The cells will then be added to the wells and incubated for 60 min at room temperature. 100µl of Detection Mix containing tracer cAMP will then be added to the wells. Plates were then incubated additional 2 hours followed by counting in a Wallac MicroBeta scintillation counter. Values of cAMP/well will then be extrapolated from a standard cAMP curve which is contained within each assay plate.



Reporter-Based Assays: Cre-Luc Reporter Assay (Gs-associated receptors): macroglial cells are plated-out on 96 well plates at a density of  $2 \times 10^4$  cells per well and were transfected using Lipofectamine Reagent (BRL) the following day according to manufacturer instructions. A DNA/lipid mixture is prepared for each 6-well transfection as follows: 260ng of plasmid DNA in 100 $\mu$ l of DMEM were gently mixed with 2 $\mu$ l of lipid in 100 $\mu$ l of DMEM (the 260ng of plasmid DNA consisted of 200ng of a 8xCRE-Luc reporter plasmid, 50ng of pCMV comprising endogenous receptor or non-endogenous receptor or pCMV alone, and 10ng of a GPRS expression plasmid (GPRS in pcDNA3 (Invitrogen)). The 8XCRE-Luc reporter plasmid was prepared as follows: vector SRIF- $\beta$ -gal was obtained by cloning the rat somatostatin promoter (-71/+51) at BglV-HindIII site in the p $\beta$ gal-Basic Vector (Clontech). Eight (8) copies of cAMP response element were obtained by PCR from an adenovirus template AdpCF126CCRE8 (see, 7 Human Gene Therapy 1883 (1996)) and cloned into the SRIF- $\beta$ -gal vector at the Kpn-BglV site, resulting in the 8xCRE- $\beta$ -gal reporter vector. The 8xCRE-Luc reporter plasmid was generated by replacing the beta-galactosidase gene in the 8xCRE- $\beta$ -gal reporter vector with the luciferase gene obtained from the pGL3-basic vector (Promega) at the HindIII-BamHI site. Following 30 min. incubation at room temperature, the DNA/lipid mixture was diluted with 400  $\mu$ l of DMEM and 100 $\mu$ l of the diluted mixture was added to each well. 100  $\mu$ l of DMEM with 10% FCS were added to each well after a 4hr incubation in a cell culture incubator. The following day the transfected cells were changed with 200  $\mu$ l/well of DMEM with 10% FCS. Eight (8) hours later, the wells were changed to 100  $\mu$ l /well of DMEM without phenol red, after one wash with PBS. Luciferase activity were measured the next day using the LucLite™ reporter gene assay kit (Packard) following manufacturer instructions and read on a 1450 MicroBeta™ scintillation and luminescence counter (Wallac).

*AP1 reporter assay* (Gq-associated receptors) A method to detect Gq stimulation depends on the known property of Gq-dependent phospholipase C to cause the activation of genes containing AP1 elements in their promoter. A Pathdetect™ AP-1 cis-Reporting System (Stratagene, Catalogue # 219073) can be utilized following the protocol set forth above with respect to the CREB reporter assay, except that the components of the calcium phosphate precipitate were 410 ng pAP1-Luc, 80 ng pCMV-receptor expression plasmid, and 20 ng CMV-SEAP.

*SRF-LUC Reporter Assay* (Gq- associated receptors): One method to detect Gq stimulation depends on the known property of Gq-dependent phospholipase C to cause the activation of genes containing serum response factors in their promoter. A Pathdetect™ SRF-Luc-Reporting System (Stratagene) can be utilized to assay for Gq coupled activity in, e.g.,

COS7 cells. Cells are transfected with the plasmid components of the system and the indicated expression plasmid encoding endogenous or non-endogenous GPCR using a Mammalian Transfection™ Kit (Stratagene, Catalogue #200285) according to the manufacturer's instructions. Briefly, 410 ng SRF-Luc, 80 ng pCMV-receptor expression plasmid and 20 ng CMV-SEAP (secreted alkaline phosphatase expression plasmid; alkaline phosphatase activity is measured in the media of transfected cells to control for variations in transfection efficiency between samples) are combined in a calcium phosphate precipitate as per the manufacturer's instructions. Half of the precipitate is equally distributed over 3 wells in a 96-well plate, kept on the cells in a serum free media for 24 hours. The last 5 hours the cells are incubated with a selected compound. Cells are then lysed and assayed for luciferase activity using a Lucite™ Kit (Packard, Cat. # 6016911) and "Trilux 1450 Microbeta" liquid scintillation and luminescence counter (Wallac) as per the manufacturer's instructions. The data can be analyzed using GraphPad Prism™ 2.0a (GraphPad Software Inc.)

*Intracellular inositol 1,4,5-triphosphate (IP3) Accumulation Assay* ( $G_q$ -associated receptors): On day 1, cells comprising the receptors (endogenous and/or non-endogenous) can be plated onto 24 well plates, usually  $1 \times 10^5$  cells/well (although this number can be optimized). On day 2 cells can be transfected by firstly mixing 0.25  $\mu$ g DNA in 50  $\mu$ l serum free DMEM/well and 2  $\mu$ l lipofectamine in 50  $\mu$ l serumfree DMEM/well. The solutions are gently mixed and incubated for 15-30 min at room temperature. Cells are washed with 0.5 ml PBS and 400  $\mu$ l of serum free media is mixed with the transfection media and added to the cells. The cells are then incubated for 3-4 hrs at 37°C/5%CO<sub>2</sub> and then the transfection media is removed and replaced with 1ml/well of regular growth media.

On day 3 the cells are labeled with <sup>3</sup>H-myo-inositol. Briefly, the media is removed and the cells are washed with 0.5 ml PBS. Then 0.5 ml inositol-free/serum free media (GIBCO BRL) is added/well with 0.25  $\mu$ Ci of <sup>3</sup>H-myo-inositol/ well and the cells are incubated for 16-18 hrs o/n at 37°C/5%CO<sub>2</sub>. On Day 4 the cells are washed with 0.5 ml PBS and 0.45 ml of assay medium is added containing inositol-free/serum free media 10  $\mu$ M pargyline 10 mM lithium chloride or 0.4 ml of assay medium and 50  $\mu$ l of 10x ketanserin (ket) to final concentration of 10  $\mu$ M. The cells are then incubated for 30 min at 37°C. The cells are then washed with 0.5 ml PBS and 200  $\mu$ l of fresh/ice cold stop solution (1M KOH; 18 mM Na-borate; 3.8 mM EDTA) is added/well. The solution is kept on ice for 5-10 min or until cells were lysed and then neutralized by 200  $\mu$ l of fresh/ice cold neutralization sol. (7.5 % HCL).

The lysate is then transferred into 1.5 ml eppendorf tubes and 1 ml of chloroform/methanol (1:2) is added/tube. The solution is vortexed for 15 sec and the upper phase is applied to a Biorad AG1-X8™ anion exchange resin (100-200 mesh). Firstly, the resin

is washed with water at 1:1.25 W/V and 0.9 ml of upper phase is loaded onto the column. The column is washed with 10 mls of 5 mM myo-inositol and 10 ml of 5 mM Na-borate/60mM Na-formate. The inositol tris phosphates are eluted into scintillation vials containing 10 ml of scintillation cocktail with 2 ml of 0.1 M formic acid/ 1 M ammonium formate. The columns are  
5 regenerated by washing with 10 ml of 0.1 M formic acid/3M ammonium formate and rinsed twice with dd H<sub>2</sub>O and stored at 4°C in water.

*Fluorometric Imaging Plate Reader (FLIPR) Assay for the Measurement of Intracellular Calcium Concentration:* Target Receptor (experimental) and pCMV (negative control) stably transfected cells from respective clonal lines are seeded into poly-D-lysine  
10 pretreated 96-well plates (Becton-Dickinson, #356640) at  $5.5 \times 10^4$  cells/well with complete culture medium (DMEM with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate) for assay the next day. To prepare Fluo4-AM (Molecular Probe, #F14202) incubation buffer stock, 1 mg Fluo4-AM is dissolved in 467  $\mu$ l DMSO and 467  $\mu$ l Pluronic acid (Molecular Probe, #P3000) to give a 1 mM stock solution that can be stored at -20°C for a month. Fluo4-AM is a  
15 fluorescent calcium indicator dye.

Candidate compounds are prepared in wash buffer (1X HBSS/2.5 mM Probenicid/20 mM HEPES at pH 7.4).

At the time of assay, culture medium is removed from the wells and the cells are loaded with 100  $\mu$ l of 4  $\mu$ M Fluo4-AM/2.5 mM Probenicid (Sigma, #P8761)/20 mM HEPES/complete  
20 medium at pH 7.4. Incubation at 37°C/5% CO<sub>2</sub> is allowed to proceed for 60 min.

After the 1 hr incubation, the Fluo4-AM incubation buffer is removed and the cells are washed 2X with 100  $\mu$ l wash buffer. In each well is left 100  $\mu$ l wash buffer. The plate is returned to the incubator at 37°C/5% CO<sub>2</sub> for 60 min.

FLIPR (Fluorometric Imaging Plate Reader; Molecular Device) is programmed to add  
25 50  $\mu$ l candidate compound on the 30<sup>th</sup> second and to record transient changes in intracellular calcium concentration ([Ca<sup>2+</sup>]) evoked by the candidate compound for another 150 seconds. Total fluorescence change counts are used to determine agonist activity using the FLIPR software. The instrument software normalizes the fluorescent reading to give equivalent initial readings at zero.

30 In some embodiments, the cells comprising Target Receptor further comprise promiscuous G alpha 15/16 or the chimeric Gq/Gi alpha unit.

Although the foregoing provides a FLIPR assay for agonist activity using stably transfected cells, a person of ordinary skill in the art would readily be able to modify the assay in order to characterize antagonist activity. Said person of ordinary skill in the art would also  
35 readily appreciate that, alternatively, transiently transfected cells could be used.

It is evident from the above results and discussion that the subject invention provides an important new means for producing olfactory GPCRs. In particular, the subject invention provides a system for screening chemical agent libraries to find olfactory GPCR modulators. As  
5 such, the subject methods and systems find use in a variety of different applications, including research, food and fragrance improvement, and other applications. Accordingly, the present invention represents a significant contribution to the art.

Table 1

OR51B2	P47884	Q8VGE1	Q8VG94	Q8VG21	Q8VEZ0	Q8VGS5	Q8VGU6
Q9Y5P0	P58170	Q8VGJ1	Q8VG95	Q8VG34	Q8VEZ9	Q8VGS6	Q8VGU7
Q9H255	P30953	Q8VGJ6	Q8VGA0	Q8VG41	Q8VF01	Q8VGS7	Q8VGU8
O88628	P47887	Q8VGP8	Q8VGA9	Q8VG47	Q8VF12	Q8VGS8	Q8VGX1
Q9H343	O43749	Q8VGT6	Q8VGB1	Q8VG57	Q8VF13	Q8VGS9	Q8VGX5
Q9H344	P47890	Q8VGT7	Q8VGB2	Q8VG58	Q8VF14	Q8VGT8	Q9JHB2
Q9H341	O60431	Q8VGT9	Q8VGB6	Q8VG59	Q8VF15	Q8VGU3	Q9JHW3
Q9UKL2	Q15612	Q920G5	Q8VGB7	Q8VG60	Q8VF19	Q8VGY2	Q9JM16
Q96RD2	P23269	Q9EPG3	Q8VGC8	Q8VG61	Q8VF22	Q8VH07	O35184
Q9H346	P23274	Q9EPG4	Q8VGD6	Q8VG62	Q8VF25	Q8VH08	Q96RD0
Q96RD3	P23273	Q9JKA6	Q8VGD7	Q8VG63	Q8VF34	Q8VH10	Q96RC9
Q8NGF0	P23266	Q9GZK7	Q8VGD8	Q8VG73	Q8VF36	Q920P1	Q15620
Q8NGF1	P23271	Q8NG94	Q8VGD9	Q8VG74	Q8VF50	Q920P2	Q8WZ84
Q8NGF3	P23272	Q8NGC1	Q8VG10	Q8VG82	Q8VF51	Q923Q6	Q9GZM6
Q8NGH5	P30955	Q8NGC7	Q8VGJ5	Q8VG86	Q8VF52	Q923Q8	Q63395
Q8NGH6	P70526	Q8NGC9	Q8VGL6	Q8VGE4	Q8VF53	Q9EQ84	Q8N0Y1
Q8NGH7	Q62942	Q8NH07	Q8VGL7	Q8VGE5	Q8VF54	Q9EQ85	Q8NG78
Q8NGH8	Q8NGA1	Q8VEV3	Q8VGP4	Q8VGE6	Q8VF59	Q9EQ86	Q8NG88
Q8NGH9	Q8NGQ3	Q8VEV4	Q8VGP5	Q8VGE7	Q8VF60	Q9EQ87	Q8NGG6
Q8NGI0	Q8NGR2	Q8VF70	Q8VGP6	Q8VGE8	Q8VF61	Q9EQ88	Q8NGG7
Q8NGI1	Q8NGR5	Q8VFC3	Q8VGT5	Q8VGE9	Q8VF65	Q9QY00	Q8NGG8
Q8NGI2	Q8NGR7	Q8VFD8	Q8VGU0	Q8VGF1	Q8VF66	Q9WU91	Q8NGG9
Q8NGI3	Q8NGR8	Q8VFE3	Q8VGW6	Q8VGF2	Q8VF67	O13036	Q8NGH0
Q8NGJ2	Q8NGR9	Q8VFT6	Q8VGW9	Q8VGF3	Q8VF68	O57597	Q8NGH1
Q8NGJ3	Q8NGS0	Q8VFT7	Q8VGX0	Q8VGF4	Q8VF71	O95222	Q8NGH2
Q8NGJ4	Q8NGS1	Q8VFT8	Q8VGX2	Q8VGF5	Q8VF72	O95007	Q8NGM9
Q8NGJ5	Q8NGS2	Q8VFT9	Q920Z2	Q8VGF6	Q8VF73	O70269	Q8VEY0
Q8NGJ6	Q8NGS3	Q9WU86	Q9D3U9	Q8VGF7	Q8VF74	O70270	Q8VF23
Q8NGJ7	Q8NGZ1	P58182	Q9D4F9	Q8VGF8	Q8VF75	O70271	Q8VF62
Q8NGJ8	Q8NH92	Q9UGF7	Q9EP55	Q8VGF9	Q8VF76	P23267	Q8VF63
Q8NGJ9	Q8NH93	Q8NHA7	Q9EP67	Q8VGG0	Q8VF77	P23270	Q8VF64
Q8NGK0	Q8NH94	Q8VG96	Q9EPF5	Q8VGG1	Q8VFC4	Q8C0U2	Q8VF78
Q8NGK1	Q8NHA8	Q920Y8	Q9EPF6	Q8VGG2	Q8VFC5	Q8K4Z9	Q8VFB3
Q8NGK2	Q8VET9	Q920Y9	Q9EPF7	Q8VGG7	Q8VFC9	Q8K501	Q8VFB4
Q8NGK3	Q8VEU7	Q920Z0	Q9EPF8	Q8VGG8	Q8VFD0	Q8NGC5	Q8VFB5
Q8NGK4	Q8VEY8	O95047	Q9EPF9	Q8VGH7	Q8VFD1	Q8NGD9	Q8VFB6
Q8NGK5	Q8VEZ6	Q9GZK3	Q9EPG0	Q8VGH8	Q8VFD2	Q8NGE1	Q8VFD7
Q8NGK6	Q8VEZ7	O76000	Q9EPG5	Q8VGH9	Q8VFD3	Q8NGE2	Q8VFN2
Q8NGM7	Q8VF79	P58173	Q9EPG6	Q8VGM3	Q8VFG0	Q8NGM8	Q8VFN3
Q8NH53	Q8VFA1	O95371	Q9EPV1	Q8VGM4	Q8VFG1	Q8NGN1	Q8VFN4
Q8NH55	Q8VFD9	Q9H210	Q9GZK1	Q8VGM5	Q8VFG5	Q8NGQ2	Q8VFN5
Q8NH56	Q8VFE0	Q13607	Q9GZK6	Q8VGM6	Q8VFG6	Q8NGT5	Q8VG15
Q8NH57	Q8VFE1	O95006	Q9QW34	Q8VGM7	Q8VFJ7	Q8NGU2	Q8VG16
Q8NH60	Q8VFE4	Q9H205	Q9QW38	Q8VGM8	Q8VFJ8	Q8NGW0	Q8VG17
Q8NH61	Q8VFE5	Q9GZK4	Q9QZ17	Q8VGM9	Q8VFJ9	Q8NGW1	Q8VG50
Q8NH63	Q8VFE6	O95918	Q9QZ18	Q8VGN0	Q8VFK0	Q8NGW6	Q8VG51

Table 1

Q8NH64	Q8VFM9	Q15062	Q9QZ19	Q8VGN1	Q8VFK1	Q8NGX0	Q8VG52
Q8NH67	Q8VFP4	O76002	Q9QZ20	Q8VGN2	Q8VFK2	Q8NGX8	Q8VG53
Q8NH68	Q8VFP5	O76001	Q9QZ21	Q8VGN3	Q8VFK3	Q8NGX9	Q8VG54
Q8NH76	Q8VFP6	Q9NQN1	Q9QZ22	Q8VGN4	Q8VFK4	Q8NGY2	Q8VG55
Q8NH78	Q8VFP7	O43869	Q9R0Z2	Q8VGN5	Q8VFK5	Q8NGY3	Q8VG56
Q8TCB6	Q8VFP8	Q9Y3N9	P47881	Q8VGN6	Q8VFK6	Q8NGY4	Q8VG67
Q8VBV9	Q8VFP9	O35434	P47893	Q8VGN7	Q8VFK7	Q8NGY5	Q8VG68
Q8VEW7	Q8VFT2	O95499	P47888	Q8VGN8	Q8VFK8	Q8NGY6	Q8VG69
Q8VEW8	Q8VFY1	P23275	P47883	Q8VGN9	Q8VFK9	Q8NGZ6	Q8VG70
Q8VEX9	Q8VGB9	Q95156	Q8VFX6	Q8VGP0	Q8VFL0	Q8NH40	Q8VG71
Q8VF02	Q8VGG9	Q63394	Q8VFX7	Q8VGP1	Q8VFL1	Q8NH79	Q8VG75
Q8VF03	Q8VGH0	Q8N349	Q8VFX8	Q8VGP2	Q8VFL2	Q8VEU0	Q8VG76
Q8VF06	Q8VGH1	Q8N628	Q8VFX9	Q8VGP3	Q8VFL4	Q8VEU1	Q8VG80
Q8VF07	Q8VGI1	Q8NG76	Q8VGR1	Q9QW37	Q8VFL5	Q8VEW0	Q8VG89
Q8VF08	Q8VGI2	Q8NG77	Q8VGR2	Q9R0K1	Q8VFL6	Q8VEX2	Q8VG90
Q8VF09	Q8VGI3	Q8NG80	Q9TSM7	Q9R0K2	Q8VFL7	Q8VEX8	Q8VG92
Q8VF27	Q8VGJ7	Q8NG81	Q9TSM8	Q9R0K3	Q8VFL8	Q8VF24	Q8VG93
Q8VF28	Q8VGJ8	Q8NG82	Q9TU88	Q9R0K4	Q8VFL9	Q8VF26	Q8VGB4
Q8VFZ7	Q8VGJ9	Q8NG83	Q9TU89	Q9R0K5	Q8VFM0	Q8VF30	Q8VGC9
Q8VG01	Q8VGK0	Q8NG84	Q9TU97	Q96R09	Q8VFM1	Q8VF31	Q8VGD0
Q8VG18	Q8VGK1	Q8NG85	Q9TUA0	Q96R08	Q8VFN7	Q8VF33	Q8VGD1
Q8VG19	Q8VGK2	Q8NG86	Q9TUA4	O95221	Q8VFN8	Q8VF82	Q8VGD2
Q8VG22	Q8VGK3	Q8NG97	Q15615	Q13606	Q8VFN9	Q8VFB7	Q8VGD3
Q8VG23	Q8VGK4	Q8NGH3	P58180	Q8WZ92	Q8VFP1	Q8VFE7	Q8VGD4
Q8VG24	Q8VGK5	Q8NGH4	O95013	Q8WZ94	Q8VFP3	Q8VFH3	Q8VGD5
Q8VG25	Q8VGK6	Q8NGS4	Q8LXE1	Q9UGF5	Q8VFP4	Q8VFH4	Q8VGE2
Q8VG26	Q8VGK7	Q8NGS5	Q8K4Z8	Q9UGF6	Q8VFP5	Q8VFH5	Q8VGE3
Q8VG28	Q8VGK8	Q8NGS6	Q8K500	O77756	Q8VFP6	Q8VFH6	Q8VH09
Q8VG77	Q8VGK9	Q8NGS7	Q8N0Y3	O77757	Q8VFP7	Q8VFH7	Q9EQ89
Q8VG78	Q8VGL0	Q8NGS8	Q8NGA8	O77758	Q8VFP8	Q8VFH8	Q9EQ90
Q8VG79	Q8VGP7	Q8NGS9	Q8NGB1	Q95154	Q8VFP9	Q8VFH9	Q9EQ91
Q8VG84	Q8VGR3	Q8NGT0	Q8NGB2	P37067	Q8VFR0	Q8VFI0	Q9EQ92
Q8VG85	Q8VGR4	Q8NGT1	Q8NGB4	Q95155	Q8VFR1	Q8VFI1	Q9EQ93
Q8VGA1	Q8VGR5	Q8NGT2	Q8NGB6	P37068	Q8VFR2	Q8VFI2	Q9EQ94
Q8VGU9	Q8VGR6	Q8NGT6	Q8NGB8	P37069	Q8VFR3	Q8VFI3	Q9EQ95
Q8VGV0	Q8VGR7	Q8NGT7	Q8NGB9	P37070	Q8VFR4	Q8VFI4	Q9EQ96
Q8VGV1	Q8VGT0	Q8NGT8	Q8NGC2	P37071	Q8VFR5	Q8VFI5	Q9EQ97
Q8VGV2	Q8VGT1	Q8NGT9	Q8NGC6	P37072	Q8VFR6	Q8VFN6	Q9EQ98
Q8VGV3	Q8VGT2	Q8NGU4	Q8NGD0	Q62943	Q8VFR7	Q8VFP0	Q9EQ99
Q8VGV4	Q8VGT3	Q8NGV0	Q8NGD1	Q62944	Q8VFR8	Q8VFP2	Q9EQA0
Q8VGV5	Q920Y6	Q8NGV1	Q8NGD2	Q8C0S2	Q8VFR9	Q8VFU0	Q9EQA1
Q8VGV6	Q920Y7	Q8NGV4	Q8NGD3	Q8IVL3	Q8VFS0	Q8VFU1	Q9EQA2
Q8VGV8	Q9JHE2	Q8NGV5	Q8NGD4	Q8LXE7	Q8VFS7	Q8VFU2	Q9EQA3
Q8VGV9	Q9QW35	Q8NGW7	Q8NGD5	Q8N0Y5	Q8VFS8	Q8VFU5	Q9EQA4
Q8VGW0	Q9TQX4	Q8NGX1	Q8NGD6	Q8N127	Q8VFS9	Q8VFY9	Q9EQA5
Q8VGW1	Q9TSN0	Q8NGX2	Q8NGE8	Q8N146	Q8VFT0	Q8VFZ0	Q9EQA6

Table 1

Q8VGW2	Q9TU84	Q8NGY9	Q8NGF8	Q8N162	Q8VFU3	Q8VFZ1	Q9EQA7
Q8VGW3	Q9TU86	Q8NGZ0	Q8NGF9	Q8NG75	Q8VFU4	Q8VFZ2	Q9EQA8
Q8VGW4	Q9TU90	Q8NGZ4	Q8NGI4	Q8NGC0	Q8VFU6	Q8VFZ8	Q9EQA9
Q8VGW5	Q9TU92	Q8NGZ5	Q8NGI6	Q8NGC3	Q8VFU7	Q8VFZ9	Q9EQB0
Q8VGX3	Q9TU93	Q8NGZ9	Q8NGJ1	Q8NGC4	Q8VFX2	Q8VG27	Q9EQB1
Q8VGX4	Q9TU94	Q8NH00	Q8NGL6	Q8NGE7	Q8VFX3	Q8VG29	Q9EQB2
Q8VGX6	Q9TU95	Q8NH01	Q8NGL7	Q8NGE9	Q8VFX4	Q8VG33	Q9EQB3
Q8VGX7	Q9TU99	Q8NH02	Q8NGL8	Q8NGF4	Q8VFX5	Q8VG45	Q9EQB4
Q8VGX8	Q9TUA1	Q8NH04	Q8NGL9	Q8NGF5	Q8VFX6	Q8VG46	Q9EQB5
Q8VGX9	Q9TUA2	Q8NH16	Q8NGM0	Q8NGF7	Q8VFX7	Q8VG64	Q9EQB6
Q8VGY0	Q9TUA3	Q8NH95	Q8NGN0	Q8NGG0	Q8VFX8	Q8VGC2	Q9EQB7
Q8VGY1	Q9TUA6	Q8NHA4	Q8NGN8	Q8NGG2	Q8VFX9	Q8VGC4	Q9EQB8
Q8VGY3	Q9TUA7	Q8NHA6	Q8NGN9	Q8NGG3	Q8VFW0	Q8VGC5	Q9EQG1
Q8VGY4	Q9TUA8	Q8NHC8	Q8NGP0	Q8NGG4	Q8VFW1	Q8VGH3	Q9ERU6
Q8VGY5	Q9TUA9	Q8VES9	Q8NH05	Q8NGG5	Q8VFW2	Q8VGH4	Q9QW36
Q8VGY6	Q9UDD9	Q8VET2	Q8NH21	Q8NGI8	Q8VFW3	Q8VGH5	Q8N148
Q8VGY7	O70265	Q8VEV0	Q8NH37	Q8NGI9	Q8VFW4	Q8VGH6	Q8NG79
Q8VGY8	O70266	Q8VEV1	Q8NH41	Q8NGJ0	Q8VFW5	Q8VGI7	Q8NG92
Q8VGY9	O70267	Q8VEV9	Q8NH42	Q8NGK9	Q8VFW6	Q8VGI8	Q8NGE0
Q8VGZ0	O70268	Q8VEW4	Q8NH43	Q8NGL0	Q8VFW7	Q8VGI9	Q8NGR1
Q8VGZ1	P58181	Q8VEW9	Q8NH49	Q8NGL1	Q8VFW8	Q8VGJ0	Q8NGR6
Q8VGZ2	Q9H209	Q8VEY4	Q8NH70	Q8NGL2	Q8VFW9	Q8VGJ2	Q8NGV6
Q8VGZ3	Q9H207	Q8VEY6	Q8NH72	Q8NGL3	Q8VFX0	Q8VGJ3	Q8NGV7
Q8VGZ4	Q96KK4	Q8VEY7	Q8NH73	Q8NGL4	Q8VFX1	Q8VGL1	Q8NGZ3
Q8VGZ5	Q9Y4A9	Q8VF05	Q8NH83	Q8NGL5	Q8VFX2	Q8VGU1	Q8NH08
Q8VGZ6	O60403	Q8VF17	Q8NH84	Q8NGN2	Q8VFX3	Q8VGU4	Q8NH09
Q8VGZ7	O60404	Q8VF18	Q8VET0	Q8NGN3	Q8VFX4	Q8VGU5	Q8NH14
Q8VGZ8	P30954	Q8VF37	Q8VET4	Q8NGN4	Q8VFX5	Q8VGW8	Q8NH44
Q8VGZ9	Q62007	Q8VF44	Q8VEX0	Q8NGN5	Q8VFZ3	Q924H8	Q8NHB7
Q8VH00	Q8CG22	Q8VF69	Q8VEX1	Q8NGN6	Q8VG00	Q9EPG1	Q8NHB8
Q8VH01	Q8NGA5	Q8VF80	Q8VEX3	Q8NGN7	Q8VG02	Q9EPG2	Q8NHC5
Q8VH02	Q8NGA6	Q8VF81	Q8VEX7	Q8NGP2	Q8VG03	Q9EPV0	Q8NHC6
Q8VH03	Q8NGE3	Q8VF87	Q8VEY5	Q8NGP3	Q8VG04	Q9H206	Q8VET6
Q8VH04	Q8NGE5	Q8VF88	Q8VEZ1	Q8NGP4	Q8VG05	Q9QWU6	Q8VET7
Q8VH05	Q8NGF6	Q8VF89	Q8VEZ2	Q8NGP6	Q8VG06	Q9Z1V0	Q8VEX5
Q8VH06	Q8NGI7	Q8VF92	Q8VEZ3	Q8NGP8	Q8VG07	P34987	Q8VEX6
Q8VH11	Q8NGM4	Q8VFA2	Q8VF10	Q8NGP9	Q8VG08	Q15622	Q8VF04
Q8VH12	Q8NGQ4	Q8VFA3	Q8VF11	Q8NGQ0	Q8VG09	O76100	Q8VF16
Q8VH13	Q8NGX3	Q8VFA4	Q8VF21	Q8NGQ1	Q8VG11	O14581	Q8VF32
Q8VH14	Q8NGX5	Q8VFA5	Q8VF29	Q8NGQ5	Q8VG13	O76099	Q8VF35
Q8VH15	Q8NGX6	Q8VFA6	Q8VF38	Q8NGQ6	Q8VG20	O60412	Q8VF42
Q8VH16	Q8NGY0	Q8VFA7	Q8VF39	Q8NGR3	Q8VG30	P23268	Q8VF43
Q8VH17	Q8NGY1	Q8VFA8	Q8VF40	Q8NGR4	Q8VG35	P23265	Q8VF93
Q8VH18	Q8NH19	Q8VFA9	Q8VF41	Q8NGZ2	Q8VG36	Q95157	Q8VFB8
Q8VH19	Q8NH36	Q8VFB2	Q8VF45	Q8NH10	Q8VG37	Q8N133	Q8VFB9
Q8VH20	Q8NH74	Q8VFC1	Q8VF46	Q8NH18	Q8VG38	Q8NG95	Q8VFC0

Table 1

Q8VH21	Q8NHC4	Q8VFC2	Q8VF47	Q8NH48	Q8VG39	Q8NG98	Q8VFE8
Q8VH22	Q8VBW9	Q8VFD4	Q8VF48	Q8NH50	Q8VG40	Q8NG99	Q8VFE9
Q924X8	Q8VES6	Q8VFD5	Q8VF56	Q8NH51	Q8VG42	Q8NGA0	Q8VFP3
Q99NH4	Q8VES7	Q8VFD6	Q8VF57	Q8NH69	Q8VG43	Q8NGA2	Q8VFX0
Q9EPN8	Q8VEU3	Q8VFF0	Q8VF58	Q8NH80	Q8VG44	Q8NH99	Q8VFX6
Q9EPN9	Q8VEV2	Q8VFG2	Q8VF83	Q8NH81	Q8VG65	Q8NHB5	Q8VFX7
Q9EQQ5	Q8VEW1	Q8VFG3	Q8VF84	Q8NH85	Q8VG66	Q8NHC1	Q8VG48
Q9EQQ6	Q8VEX4	Q8VFG4	Q8VF85	Q8NH86	Q8VG81	Q8VET8	Q8VGH2
Q9EQQ7	Q8VEY1	Q8VFG7	Q8VF86	Q8NH87	Q8VG83	Q8VEW3	Q8VGJ4
Q9GKV8	Q8VEZ4	Q8VFG8	Q8VF90	Q8NH88	Q8VG91	Q8VEY9	Q8VGL2
Q9H2C5	Q8VEZ5	Q8VFG9	Q8VF91	Q8NH89	Q8VG97	Q8VFF1	Q8VGL3
Q9H2C6	Q8VEZ8	Q8VFH0	Q8VF94	Q8NH90	Q8VGA2	Q8VFF2	Q8VGL4
Q9H2C8	Q8VF00	Q8VFH1	Q8VF95	Q8NH91	Q8VGA3	Q8VFF3	Q8VGL5
Q9H339	Q8VF20	Q8VFH2	Q8VF96	Q8NHC7	Q8VGA4	Q8VFF4	Q8VGL8
Q9H340	Q8VF55	Q8VFL3	Q8VF97	Q8VES8	Q8VGA5	Q8VFF5	Q8VGL9
Q9H342	Q8VFE2	Q8VFM2	Q8VF98	Q8VET1	Q8VGA6	Q8VFF6	Q8VGM0
Q9H345	Q8VFM7	Q8VFM3	Q8VF99	Q8VET3	Q8VGA7	Q8VFF7	Q8VGM1
Q9WU88	Q8VFQ0	Q8VFM4	Q8VFA0	Q8VET5	Q8VGA8	Q8VFI7	Q8VGM2
Q9WU89	Q8VFQ2	Q8VFM5	Q8VFB0	Q8VEU2	Q8VGB0	Q8VFI8	Q8VGP9
Q9WU90	Q8VFS1	Q8VFM6	Q8VFB1	Q8VEU4	Q8VGB3	Q8VFJ0	Q8VGQ0
Q9WU93	Q8VFT1	Q8VFN0	Q8VFC6	Q8VEU5	Q8VGB5	Q8VFJ1	Q8VGQ1
Q9WU94	Q8VFX4	Q8VFQ1	Q8VFC7	Q8VEU6	Q8VGC6	Q8VFJ2	Q8VGQ2
Q9WVD7	Q8VFX5	Q8VFS2	Q8VFC8	Q8VEU8	Q8VGC7	Q8VFJ3	Q8VGQ3
Q9WVD8	Q8VFZ4	Q8VFS3	Q8VFF8	Q8VEU9	Q8VGF0	Q8VFJ4	Q8VGQ4
Q9WVD9	Q8VFZ5	Q8VFS4	Q8VFF9	Q8VEV5	Q8VGI4	Q8VFJ5	Q8VGQ5
Q9WVN4	Q8VFZ6	Q8VFS5	Q8VFN1	Q8VEV6	Q8VGI5	Q8VFJ6	Q8VGQ6
Q9WVN5	Q8VG10	Q8VFS6	Q8VFT3	Q8VEV7	Q8VGI6	Q8VFM8	Q8VGQ7
Q9WVN6	Q8VG31	Q8VFX2	Q8VFT4	Q8VEV8	Q8VGR8	Q8VG88	Q8VGQ8
Q9YH55	Q8VG32	Q8VFX3	Q8VFT5	Q8VEW2	Q8VGR9	Q8VGB8	Q8VGQ9
Q9P1Q5	Q8VG98	Q8VG14	Q8VFU8	Q8VEW5	Q8VGS0	Q8VGG3	Q8VGR0
Q9Y585	Q8VG99	Q8VG49	Q8VFU9	Q8VEW6	Q8VGS1	Q8VGG4	Q8VGT4
Q15619	Q8VGC0	Q8VG72	Q8VFX0	Q8VEY2	Q8VGS2	Q8VGG5	Q8VGU2
P34982	Q8VGC1	Q8VG87	Q8VFX1	Q8VEY3	Q8VGS3	Q8VGG6	Q8VGV7
	Q8VGE0		Q8VG12		Q8VGS4		Q8VGW7